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THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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CONSERVATION OF SCHOLARLY JOURNALS

The American Library Association created this last year the Committee on Aid to Libraries in War Areas, headed by John R. Russell, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the value of particular periodicals to the project should be directed to Wayne M. Hartwell, Executive Assistant to the Committee on Aid to Libraries in War Areas, Rush Rhees Library, University of Rochester, Rochester, New York.

THE BIOLOGICAL BULLETIN

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PHYSIOLOGICAL OBSERVATIONS UPON A LARVAL EUSTRONGYLIDES

II. THE AEROBIC RESPIRATION ¹

THEODOR VON BRAND ²

In the first paper of this series (von Brand, 1938) the chemical composition of a larval *Eustrongylides* was studied to some extent. It was characterized by a high glycogen and haemoglobin content, whereas only a little fat was found. The different rates of glycogen consumption under aerobic and anaerobic conditions seemed to indicate that the worm, in contrast to *Ascaris* and several other parasitic worms, was adapted to an oxidative rather than an anoxidative type of metabolism. In order to carry the analysis of its metabolic processes one step farther, a study of its respiratory exchange was undertaken.

MATERIAL AND METHODS

All the larvae (probably the larva of *Eustrongylides ignotus* according to a personal communication by Dr. B. G. Chitwood) were extracted from cysts along the mesenteries of *Fundulus heteroclitus* and only medium-sized to large worms (40 to about 120 mgm.) were used. They were washed with saline, dried quickly on filter paper, weighed with an accuracy of 1 mgm. and placed in the respiratory vessels. From one to four worms (100 to 300 mgm. fresh tissue) were used in making a single determination. All the data given below for the gaseous exchange are based on the weight of the living worms immediately after isolation. The salt solutions used varied in the different series, and are mentioned below.

The experiments were carried out with manometers of the Warburg type, using vessels of about 18 cc. capacity, and conducted at a temperature of 37° C. This high temperature was chosen rather than the lower

¹ A contribution from the Department of Biology, The Catholic University of America, Washington, D. C.

² The author is indebted to the Elizabeth Thompson Science Fund for a grant towards the purchase of the respiration apparatus used in this investigation.



temperature to which the worms were accustomed in the fish, since the definitive host is quite certainly a warm-blooded animal and the parasite would normally live at the temperature prevailing in the glands of the fore-stomach of aquatic birds after leaving the cyst. To determine the respiratory quotients of the experiments summarized in Tables III and IV, the worms were kept first for two hours in the vessels without KOH, then for the same length of time in the same vessels after addition of KOH. This procedure was permissible, since preliminary experiments had shown that the manometer readings, both for CO_2 and O_2 , remained sufficiently constant during such periods. The respiratory quotients of the experiments summarized in Table V, on the other hand, were determined according to Warburg's indirect method, since it was necessary to determine here the RQ's in shorter intervals throughout the course of the experiments. In all cases the readings were taken in half-hour intervals, and the first reading was made 20 minutes after the vessels were inserted in the water-bath.

The readings were taken to the nearest 0.5 mm. The average change in the manometer level was about 8 mm. between two readings in the oxygen consumption experiments. The average error of the single determination from this source alone may, therefore, have been as high as ± 6 per cent, or even higher, if a similar error for the thermobarometer was added. In the determination of the R.Q., obviously an accumulation of errors occurs; for example, the use of different batches of animals for the determination of the oxygen consumption and carbon dioxide production in those series in which Warburg's indirect method was employed might result in reading errors on three manometers (two for the animals and one for the thermobarometer) besides the biological variation. The exact limits of error of the R. Q. determinations are difficult to evaluate. The "extremes" given in the tables doubtless do not represent the true range of biological variability, but are a summation of this and the errors. The conclusions drawn in this paper are, therefore, based on mean values of a number of experiments for each series, as indicated in the tables. The similarities of the mean values of series conducted under comparable conditions are regarded as sufficient proof that the errors inherent in the single determinations have largely been eliminated and that large differences in the mean values of experiments conducted under different conditions are real.

RESULTS

It was noticed in experiments conducted in another connection that the worms showed *in vitro* a surprising resistance against changes in the osmotic pressure of their surroundings. It seemed therefore of interest

to study the oxygen consumption in various NaCl concentrations (Table I). The solutions used varied from distilled water to 4 per cent NaCl and with each solution seven individual experiments were performed, each lasting four hours. The initial mean values were fairly similar in all series. They were always somewhat higher than those found in the later stages of the experiments, an observation that will be discussed below. It is apparent that beginning with the third or fourth half-hour period a steady stage was reached in the lower concentrations (0–1.5 per cent NaCl), whereas in the higher concentrations (2.0–4.0 per cent NaCl) a more or less continuous decline occurred. The values of the different series belonging to these two groups have been averaged and the resulting curves are shown in Fig. 1. The steady decline of the O_2 consumption in the higher salt concentrations is obviously an indica-

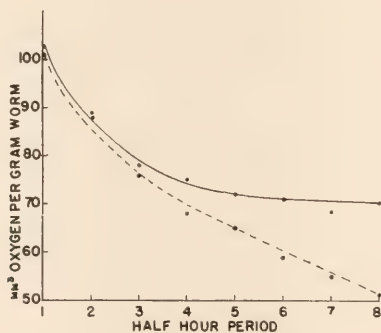


FIG. 1. Oxygen consumption in salines of low and high concentration, mean values. Solid line: 0 to 1.5 per cent NaCl. Broken line: 2 per cent to 4 per cent NaCl.

tion that they are definitely toxic. The fact, however, that variations between 0 and 1.5 per cent NaCl seem not to interfere with the normal respiratory activities, is of interest. This opens up the possibility of studying in future experiments the influence of various substances upon the oxygen consumption irrespective of their osmotic properties.

The fact that these parasites are able to maintain a constant level of metabolism in media of different osmotic pressure has probably a biological basis. Although the life cycle of this species is not known in detail, it can be assumed that the worms live at various stages in surroundings differing, perhaps widely, in molecular concentration.

It should be noted that the variations in the rate of oxygen consumption between different experiments, as indicated by the "extremes" of Table I, were fairly large. Even in the highest salt concentrations, however, some worms were able to maintain a high and fairly regular rate of oxygen consumption. The surprising tolerance of single speci-

mens to a great variety of different media, as judged by their survival in vitro, was frequently noticed and will be discussed in a future paper.

The average oxygen consumption of normal worms was about 140 cu. mm. per gram per hour. It was therefore about twice as great as that reported for *Ascaris* (von Brand, 1934; Harwood and Brown, 1934; Krüger, 1936), but considerably lower than those found in *Setaria equina* (Toryu, 1934) or the hookworm (Harwood and Brown, 1934).

TABLE I

Oxygen consumption of freshly isolated worms kept in salines of different concentrations. Oxygen in cu. mm. per gram of worm. Each mean value is derived from 7 determinations.

Half-hour periods	1	2	3	4	5	6	7	8
0% NaCl								
Mean	94	95	75	83	70	68	71	70
Extremes	51-127	73-112	56-94	56-103	56-88	37-84	55-79	55-83
0.5% NaCl								
Mean	99	80	72	71	73	63	72	78
Extremes	59-130	56-117	40-104	32-123	32-117	21-136	40-143	32-149
1.0% NaCl								
Mean	117	92	81	78	77	80	66	70
Extremes	99-165	46-172	46-178	41-148	48-118	46-137	41-90	40-92
1.5% NaCl								
Mean	103	87	84	67	69	71	64	63
Extremes	63-148	63-134	45-118	28-90	36-92	36-104	27-85	27-89
2.0% NaCl								
Mean	92	82	82	60	60	60	51	55
Extremes	53-161	63-114	39-104	47-82	49-71	49-67	29-65	49-62
3.0% NaCl								
Mean	105	87	86	82	61	63	59	51
Extremes	74-170	60-119	60-112	34-134	17-112	17-100	17-100	17-99
4.0% NaCl								
Mean	105	97	91	61	73	54	56	46
Extremes	83-133	77-109	39-117	19-107	19-115	7-80	14-91	7-83

Unfortunately, a comparison with Fenwick's (1938) data for *Ascaris* larvae is impossible, since he bases his figures on numbers of larvae instead of their weight. Stannard, McCoy, and Latchford (1938) found in *Trichinella* larvae an oxygen consumption of 1.70 cu. mm. per hr. per milligram of dry weight. A calculation of the above *Eustrongylides* figures on this basis, using the previously found figure of 25 per cent dry weight (von Brand, 1938), yields a value of 0.56 cu. mm. Krüger (1940) showed that the rate of oxygen consumption of *Ascaris lumbrici*

coides of various sizes depends rather on surface than on weight. In Table II an analysis of the oxygen consumption of *Trichinella* larvae, *Eustrongylides* larvae and adult females of *Ascaris lumbricoides* has been attempted on this basis. It should be noted that no accurate figures for the fresh weight of *Trichinella* larvae are available in the literature, only data on the dry weight. The fresh weight has been calculated on the basis of 5 per cent dry weight. The conclusion drawn below remains, however, unchanged, even if the weight of the *Trichinella* larvae is calculated on the assumption of 15 per cent dry weight, a figure which is not very likely for these delicate worms. It appears that, contrary to Krüger's findings on individuals of one species, the oxygen consumption of nematodes of very different size is not constant if calculated on the basis

TABLE II

Calculation of oxygen consumption of various helminths on the basis of weight and relative surface.

Species	Weight of 1 worm in mgm.	Relative weight	Surface (Weight $\frac{2}{3}$)	Relative surface	O ₂ in cu. mm. per worm per hour	Relative O ₂ consumption
<i>Trichinella</i> larva (3)	0.007	1	0.037	1	0.0006	1
<i>Eustrongylides</i> larva (4)	82	11700	18.9	511	12.6	21000
Adult <i>Ascaris lumbricoides</i> , female (5)	4780	683000	284	7700	287	478000

³ The data of Stannard, McCoy and Latchford (1938) for oxygen consumption in saline have been used. The weight of one worm has been calculated assuming 5 per cent dry weight.

⁴ The data of the experiments conducted in 1 per cent saline (Table I) have been used.

⁵ The data of von Brand (1934) have been used.

of surface. The difference in the rates of oxygen consumption is considerably smaller, although by no means eliminated if the calculation is based on weight. It is recognized that the data are not complete enough to draw general conclusions, but the nematodes appear to be organisms exceptionally well suited for further work along these lines. Many species of intermediate size are available.

The next series was undertaken in order to ascertain the influence of starvation upon the gaseous metabolism. The worms were kept for 7 days in 1 per cent saline in an incubator at 37° C. and daily determinations of the oxygen uptake and carbon dioxide production were performed. In some instances substitute batches, that had starved a similar period under analogous conditions but for which no initial values were

TABLE III
Oxygen consumption and carbon dioxide production of worms starving in 1 per cent saline at 37° C.

Days starving	0	1	2	3	4	5	6	7
Number of determinations	9	7	8	11	11	13	5	12
O ₂ cu. mm./gram/hour Mean	152	100	113	104	74	82	85	71
Extremes	121-202	63-120	56-142	75-170	57-99	53-138	62-98	48-95
CO ₂ cu. mm./gram/hour Mean	158	101	122	113	74	82	85	75
Extremes	115-198	57-119	63-171	86-172	56-93	56-138	69-106	48-118
RQ Mean	1.07	1.02	1.06	1.10	1.01	1.01	1.01	1.04
Extremes	0.89-1.28	0.89-1.09	0.82-1.18	0.98-1.28	0.77-1.19	0.87-1.11	0.90-1.12	0.83-1.25

available, had to be used during the later days of the starvation period due to the death of worms in some of the original lots. A slow decline in the rate of oxygen consumption and carbon dioxide production occurred under the conditions of this experiment. The respiratory quotient was found to be rather variable, if individual experiments are considered. The mean values for the single days were, however, rather similar and lay always slightly above 1.0. The mean value for the whole series was 1.04 ± 0.013 , and similar mean values were also found in the series mentioned below. They are considerably lower than those reported for *Ascaris* (von Brand, 1934, Krüger, 1937) and also somewhat lower than those found in *Trichinella* larvae (Stannard, McCoy and Latchford, 1938). This indicates doubtless that the metabolism of the worm in question is mainly oxidative, but it seems likely that a small amount of anoxidative processes is superimposed on the oxidations.

In an effort to ascertain whether these surmised anoxidative processes are characterized by the production of acids, a similar series of experiments was conducted in saline containing bicarbonate. The worms were kept between determinations in 1 per cent saline at 37° C. as in the preceding series. The figures of Table IV show that the rate of respiration was somewhat higher than that of worms kept in pure saline. The mean respiratory quotient of all determinations in this series was 1.04 ± 0.019 , identical with that found in the previous series. An aerobic acid production can therefore not be demonstrated. Stannard, McCoy and Latchford (1938) found no acid production in *Trichinella* larvae kept under aerobic or anaerobic conditions. They assume the production of as yet unidentified, but non-acidic end-products. The *Eustrongylides* larvae, however, produce, as shown previously (von Brand, 1938), considerable amounts of organic acids, if kept under strictly anaerobic conditions. Naturally the question arises, whether there is such a fundamental difference between the accessory anoxidative processes proceeding under aerobic conditions and the anaerobic processes going on, if no oxygen at all is available. The above series seems to point in this direction. I do not think, however, that this is more than a possibility, hardly as yet a probability. It should be kept in mind that the CO_2 liberated by the small amounts of acids expected would not change the RQ very much. It is furthermore entirely possible that the bicarbonate reserves of the body itself are sufficient to allow neutralization of aerobically produced acids, in which case no difference between the RQ's of worms kept in media with or without bicarbonate could be expected.

The next series was designed to ascertain whether an anaerobic period previous to the determinations would have any influence upon the gaseous exchange. Freshly isolated worms were put in glass vials of about 5 cc.

TABLE IV

Oxygen consumption and carbon dioxide production of worms kept in 1 per cent saline + 0.1 per cent sodium bicarbonate at 37° C.

Days starving	0	1	2	3	4	5	6	7
Number of determinations	12	11	10	8	8	11	8	8
O ₂ cu. mm./gram/hour Mean	149	143	152	129	115	113	110	102
Extremes	106-206	94-193	106-185	97-198	80-157	77-154	45-240	59-184
CO ₂ cu. mm./gram/hour Mean	158	150	158	144	121	115	111	119
Extremes	103-276	110-201	95-228	96-214	81-188	70-158	62-252	59-357
RQ Mean	1.05	1.06	1.03	1.11	1.03	1.00	1.01	1.06
Extremes	0.84-1.38	0.89-1.21	0.89-1.23	0.94-1.35	0.91-1.16	0.74-1.15	0.93-1.11	0.75-1.94

capacity filled with 1 per cent saline of 37° C. These were then closed with a thin slice of cork, taking care to eliminate all air bubbles between the cork and the fluid by puncturing the cork with a needle and pushing it down into the fluid. A layer of mercury was then put on top of the cork, preventing the diffusion of oxygen into the vial. It was then placed for 18 to 20 hours in an incubator at 37° C. The amount of oxygen dissolved in the saline would roughly last three-quarters of an hour to

TABLE V

Gaseous exchange of worms kept previous to the determinations for 16-18 hours under anaerobic conditions ("post-anaerobic animals" in the table) and of freshly isolated worms ("fresh animals" in the table). Only the mean values are given; the variations were of the same order as those shown in Tables I, III and IV. They were somewhat more pronounced in the "post-anaerobic" than in the "fresh" lot. The O₂ series of the "fresh animals" is the same as that given in Table I for 1 per cent saline.

Half-hour periods	1	2	3	4	5	6	7	8	9	10
Number of determinations										
Post-anaerobic animals	10	10	10	10	10	10	10	10	10	6
Fresh animals	8	8	8	8	8	8	8	8	8	8
O ₂ cu. mm./gram										
Post-anaerobic animals	219	169	155	172	154	121	112	99	98	79
Fresh animals	117	92	81	78	77	80	66	70	69	74
CO ₂ cu. mm./gram										
Post-anaerobic animals	164	89	96	127	122	133	127	118	107	82
Fresh animals	107	87	86	80	85	82	72	73	73	82
RQ										
Post-anaerobic animals	0.76	0.53	0.58	0.58	0.68	1.02	1.09	1.11	1.15	0.96
Fresh animals	0.90	0.91	1.04	1.03	1.10	1.04	1.08	1.05	1.04	1.10
Excess O ₂ cu. mm./gram										
Post-anaerobic animals	145	95	74	104	77	41	46	29	29	5
Fresh animals	43	18	—	—	—	—	—	—	—	—
CO ₂ retained cu. mm./gram										
Post-anaerobic animals	68	90	68	55	41	—	—	—	—	—
Fresh animals	17	10	—	—	—	—	—	—	—	—

one hour. Added to this is an unknown amount of oxygen present in the worm tissues and combined with the haemoglobin. After about one and one-half to two hours, the color of the worms turned to the dull red of reduced haemoglobin; the actual period of anaerobiosis was therefore about 16 to 18 hours. During this time they became invariably immotile, but they began to move again shortly after restoration of aerobic conditions. Immediately after the end of the anaerobic period, the worms were placed in the respiratory vessels (Table V,

post-anaerobic animals). The rate of oxygen consumption was very high in the beginning, if compared to that of worms taken freshly from fish (Table V, fresh animals), but then went slowly down, and after 5 hours reached about the normal level. The subtraction of the oxygen values of freshly isolated worms from those of worms previously kept anaerobically reveals a total excess oxygen consumption of 584 cu. mm. per gram. To this figure must be added 61 cu. mm. of excess oxygen found in freshly isolated worms (see below), summing up to 645 cu. mm. This is probably a minimum figure which should be raised for the 20-minute equilibration period for which no data were obtainable. Assuming the same or a slightly higher excess consumption as that found in the first half-hour period, the total excess oxygen can be assumed to lie between 750 and 800 cu. mm. per gram. This excess oxygen consumption can be interpreted in two ways. It could be a purely physical process, i.e. the amount of oxygen needed to restore an equilibrium between the liquids (haemoglobin!) and tissues of the body with the surrounding fluid. In this case the process would not have much significance. The fact, however, that the bright red color of oxyhaemoglobin reappears very quickly after restoration of aerobic conditions indicates a rapid diffusion of oxygen into the body cavity. It is probable that the 20-minute equilibration period, before the first reading was taken, was sufficient to eliminate this factor.

It seems more likely that the excess oxygen consumption represents the repayment of an oxygen debt. The oxygen missed in 16 to 18 hours of anaerobiosis amounts to 2200 to 2500 cu. mm. per gram. The observed excess oxygen consumption of 750 to 800 cu. mm. represents therefore repayment of roughly 30 per cent. Obviously, then, a large part of the oxygen debt was not repaid. The process serves to remove end-products of the anaerobic metabolism. Many free-living organisms are not able to excrete these and they accumulate in the body throughout the anaerobic period. Considerable amounts of oxygen are required during the recovery period to remove them entirely, either by partial resynthesis to glycogen or by oxidation. In frog muscle, for example, a 70 per cent repayment takes place (Rotta and Stannard, 1939) and in insects even much more oxygen is used than was missed, perhaps because a larger percentage of the acids is oxidized (Gilmour, 1940, 1941). It can be safely assumed that in the present case the relatively low percentage of oxygen repayment is correlated with the fact that the worm is able to excrete at least parts of the anaerobic end-products, as evidenced by the fact that the medium becomes markedly acid during anaerobiosis (von Brand, 1938). Obviously, all excreted acids are eliminated from

participation in any recovery process. This can involve only those end-products that are still in the body when aerobic conditions are restored.

The respiratory quotients of the worms in this series were very low during the first $2\frac{1}{2}$ hours, namely 0.63 as compared to 1.06 in the following $2\frac{1}{2}$ hours. In some individual cases exceedingly low values, from 0.04 to 0.08, were observed from $\frac{1}{2}$ to $1\frac{1}{2}$ hours after the beginning of the experiments. These low respiratory quotients represent doubtless a carbon dioxide retention. The amounts of carbon dioxide retained were calculated assuming that the actual respiratory processes also had an RQ of 1.06 during the first $2\frac{1}{2}$ hours. They amounted to a total of 322 cu. mm. per gram.

It is of interest to note that the period of carbon dioxide retention was shorter than that of excess oxygen uptake ($2\frac{1}{2}$ against $4\frac{1}{2}$ to 5 hours). The mean R.Q. after the end of the carbon dioxide retention period was, as mentioned above, 1.06,—exactly the same value that was found in the control series with freshly isolated worms (Table V, fresh animals.) This indicates that the recovery processes that are superimposed upon the normal respiratory activities must have very nearly the same RQ as the latter.

It should be noted that so far very little is known about similar processes in parasitic worms. The adult *Ascaris*, which is primarily an anaerobic living organism (von Brand, 1938a) shows no accumulation of an oxygen debt during anaerobiosis (Adam, 1932; Krüger, 1936). Newly-hatched *Ascaris* larvae, on the other hand, have for a short time a distinctly increased oxygen consumption that has been interpreted as the repayment of an oxygen debt incurred during development inside the egg shell (Fenwick, 1938).

The observations described above seem to open a way to decide whether the worms lead a predominantly aerobic or anaerobic life inside the fish. If the second alternative were true, one should expect that freshly isolated worms would show the same type of oxygen consumption and similar low RQ's as worms previously subjected to anaerobic conditions in vitro. There is very little evidence that such is the case, as evidenced by the experiments summarized in Table V (fresh animals). It is true that the initial oxygen uptake was slightly higher than it was later on, a fact already mentioned above in discussing the experiments summarized in Table I. The mean respiratory quotients of the first two half-hour periods with 0.90 and 0.91 respectively were somewhat lower than that of the later periods which yielded an average of 1.06. On the basis of these figures, an excess oxygen consumption of about 61 cu. mm. per gram and a carbon dioxide retention of about 27 cu. mm. can be assumed. These are very small

figures if one considers the fact that previous to the determinations the worms had doubtless lived for months in their cysts. One would have expected the repayment of a maximal debt if the oxygen supply had been restricted noticeably. This series seems therefore to establish a sound basis for the assumption that the worms are able to satisfy very nearly their entire oxygen need inside the fish. In accordance with this view is the fact that freshly isolated worms show the bright red color of oxyhaemoglobin.

SUMMARY

1. Variations between 0 and 1.5 per cent NaCl in the medium had no influence upon the oxygen uptake of a larval *Eustrongylides*, but concentrations between 2 and 4 per cent were definitely toxic.

2. The oxygen uptake (140 cu. mm. per gram per hour) was greater than that found in *Ascaris*, but smaller than that reported for *Setaria*, hookworm or *Trichinella* larvae.

3. The oxygen uptake of nematode species of very different sizes remains more constant if calculated on the basis of weight rather than surface.

4. During a week's starvation at 37° C. the oxygen consumption decreased to about half the initial value.

5. The mean respiratory quotient of aerobically kept worms was in all series slightly above one, but no aerobic excretion of organic acids could be demonstrated with the methods employed.

6. In an aerobic period following an anaerobic period of 16 to 18 hours duration, the worms repaid about 30 per cent of the incurred oxygen debt and retained a considerable amount of carbon dioxide.

7. Freshly isolated worms, on the other hand, showed only a trace of excess oxygen consumption and carbon dioxide retainment. It is therefore concluded that the animals inside their cysts in the fish lead an almost purely oxidative life.

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THE INFLUENCE OF SURFACE ANGLE AND OF LIGHT ON THE ATTACHMENT OF BARNACLES AND OTHER SEDENTARY ORGANISMS

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INTRODUCTION

In 1935, Hopkins was able to demonstrate that more than six times as many larvae of the Pacific Coast oyster, *Ostrea lurida*, became attached to the under surface of glass plates held in a horizontal position than on the corresponding surfaces of plates at 45°, and about one hundred times as many as became attached to plates in the vertical position. The practical significance of these results to oyster growers is now well known.

The present study was undertaken to determine the behavior of other sedentary organisms in relation to plane glass surfaces held at various angles. The identification of the species involved, the number of individuals attached, and the success of colonial types, as measured by the size of the colony, could be established with precision by the use of such plates. It was hoped that, in addition to its ecological value, this study might contribute to the general problem of ship fouling.

MATERIALS AND GENERAL METHODS

Three pairs of window glass 6 by 10 inches which had been sand-blasted on both sides to give a regular but roughened surface were bound two inches apart by an encircling band of one-half inch mesh galvanized hardware cloth. Three pairs of such units were then wired to the floor and sides of a heavy wire cage which could be lowered into the sea. One pair of plate units was arranged in a horizontal position, another in a vertical, and a third at an angle of 45° to the horizontal. Considering both sides, each series of 18 plates offered surfaces at five different angles; 0° (under horizontal), 45° (under 45° plate), 90° (vertical plate—both sides), 135° (upper side of 45° plate), 180° (upper side of horizontal plate). In such series a total of 360 square inches of surface was available for the attachment of sedentary organisms at each angle excepting at 90°, where twice this amount was present. The values at this angle, therefore, were regularly halved in the presenting of data in this study.

Series of plates were placed in the sea at two points close to the Bureau of Fisheries Laboratory at Pensacola, Florida. Location 1 was the *north dock* of the island facing the open water of Santa Rosa Sound. This station was characterized by an active current and wave action. The plates were kept at a mean level of 9 feet below the surface and this was about 2 feet above the bottom. Location 2 was the *boat slip* at the southwestern end of the island. Here the current was mild and the wave action generally slight. The heavy wire cages containing the units of plates were placed directly on the sea floor so that the lowest plates were 8 inches from the bottom and the top plates were covered by about a foot of water at low tide.

After various periods of residence in the sea, plates were washed gently with fresh water to remove excess silt and salt and then dried. Counts of the organisms attached were made with the aid of a dissecting microscope.

Identifications of the Bryozoa were made by Dr. Raymond C. Osburn of Ohio State University, to whom the authors wish to express their thanks.

The set-up and the technique followed in this study were essentially those devised by Dr. A. E. Hopkins. We are deeply indebted for his friendly assistance and suggestions. Appreciation is also acknowledged to the Bureau of Fisheries for the use of laboratory facilities at Pensacola, Florida.

RESULTS OBTAINED FOR *ACANTHODESIA TENUIS* AND *ELECTRA* *HASTINGSEAE*

Sand-blasted plates maintained at the *boat slip* station accumulated relatively few barnacles but were characterized by a heavy growth of Bryozoa, particularly the encrusting species *Acanthodesia tenuis* and *Electra hastingseae* (see Tables I and II). Counts were made of the individual number of colonies for each species attached to the glass surfaces held at the various angles. Colonies of *A. tenuis* were always more numerous than *E. hastingseae* and the size of populations was always greater for plates kept in the sea for the longer period. More than 90 per cent of all Bryozoa studied were found attached to plates held at 0° and 45°. This was true for both time intervals studied. At 0° practically no portion of the plate remained uncovered after 37 days exposure.

In addition to counting the number of individual colonies, an attempt was made to measure the relative "success" of the attached larvae in relation to the angle of orientation. Planimeter measurements were

TABLE I

Twenty-three Day Plates (5/12/40 to 6/4/40)

Number of individuals and area in square centimeters of bryozoan colonies attached to sand-blasted plates in boat slip.

<i>A. tenuis</i>					<i>E. hastingsae</i>			Bar-nacles	Bi-valves	Hy-droids
	Number of colonies	Total area of colonies	Average area of colonies	Number of colonies	Total area of colonies	Average area of colonies				
0°	1.	74	245.7	3.32	20	19.3	0.97	47	29	4
	2.	91	297.5	3.27	12	15.6	1.30	170	136	7
		165	543.2	3.29	32	34.9	1.09	217	165	11
45°	1.	59	208.9	3.54	25	16.7	0.67	9	6	2
	2.	66	129.2	1.96	26	15.9	0.61	14	20	2
		125	338.1	2.71	51	32.6	0.64	23	26	4
90°	1.	4	1.1	0.28	0	0.0	0.00	1	0	0
	2.	5	1.2	0.24	1	0.2	0.20	4	0	1
		9	2.3	0.26	1	0.2	0.20	5	0	1
90°	1.	2	0.2	0.10	2	0.4	0.20	14	1	0
	2.	3	1.2	0.40	2	1.0	0.50	19	1	1
		5	1.4	0.28	4	1.4	0.35	33	2	1
135°	1.	3	1.1	0.37	1	0.1	0.10	1	0	0
	2.	0	0.0	0.00	2	0.2	0.10	3	0	1
		3	1.1	0.37	3	0.3	0.10	4	0	1
180°	1.	5	0.6	0.12	0	0.0	0.00	1	1	0
	2.	1	0.1	0.10	0	0.0	0.00	4	1	2
		6	0.7	0.12	0	0.0	0.00	5	2	2

therefore taken at the margin of each colony. A minor source of error introduced as a result of occasional over-riding edges was further complicated by the physical limitation for the spread of individuals on plates at 0° due to the density of the population. The total area and the average area of the two species of Bryozoa under discussion yielded results which resembled those found for the relation between the number of colonies and the angular orientation. That is, not only were there more individual colonies at 0° and 45°, but the ancestrulae attached on plates in these positions were more successful in giving rise to zooids.

TABLE II

Thirty-seven Day Plates (4/28/40 to 6/4/40)

Number of individuals and area in square centimeters of bryozoan colonies attached to sand-blasted plates in boat slip.

	<i>A. tenuis</i>			<i>E. hastingseae</i>			Bar-nacles	Bi-valves	Hy-droids
	Number of colonies	Total area of colonies	Average area of colonies	Number of colonies	Total area of colonies	Average area of colonies			
0°	1. 104	752.0	7.23	37	97.0	2.62	45	15	5
	2. 106	795.0	7.50	45	72.7	1.62	171	25	0
	210	1547.0	7.37	82	169.7	2.07	216	40	5
45°	1. 62	585.9	9.45	23	40.1	1.74	9	8	1
	2. 61	377.1	6.18	48	71.9	1.50	16	2	0
	123	963.0	7.83	71	112.0	1.58	25	10	1
90°	1. 5	19.3	3.86	2	9.6	4.80	2	0	0
	2. 9	14.5	1.61	6	1.0	0.17	0	0	0
	14	33.8	2.41	8	10.6	1.33	2	0	0
90°	1. 3	8.0	2.67	4	4.6	1.15	4	0	0
	2. 7	17.9	2.56	1	2.5	2.50	7	0	1
	10	25.9	2.59	5	7.1	1.42	11	0	1
135°	1. 2	0.4	0.20	0	0.0	0.00	6	0	0
	2. 4	1.2	0.30	0	0.0	0.00	14	0	0
	6	1.6	0.27	0	0.0	0.00	20	0	0
180°	1. 0	0.0	0.00	0	0.0	0.00	0	0	0
	2. 2	0.3	0.15	0	0.0	0.00	0	0	0
	2	0.3	0.15	0	0.0	0.00	0	0	0

POPULATIONS ON PLATES AT KNOWN ANGLES EXPOSED FOR FIFTEEN DAYS

Counts were made of seven groups of organisms which had become attached to a series of plates held at 0°, 45°, 90°, 135°, and 180° placed at the *north dock* for a period of 15 days (5/31/40 to 6/15/40); 19,751 individuals were found for the total area of 1800 square inches (see Table III).

Characteristically, the position designated as 0° (under surface of plates in the horizontal position) and 45° supported the largest number of individuals of all types with the exception of the ivory barnacle,

Balanus eburneus. The behavior of this organism will be discussed in another section of this study. In contrast to plates exposed in the *boat slip* for 23 days and for 37 days during approximately the same time, the 15-day plates at the *north dock* carried a total population which was more than twice as large. Colonies of *A. tenuis* though very numerous were made up of very few individuals. Plates at 0° were so densely covered with organisms that the counts reported probably represent the maximal limits of populations possible under conditions of extreme overcrowding. Coe (1932) has noted that numerous young may seriously interfere with early settlers by depriving the latter of food and oxygen.

Colonies of *E. hastingsae* were not numerous although it must be stated that identification of recently attached Bryozoa is difficult and

TABLE III

Fifteen-Day Series (5/31/40 to 6/15/40)

Distribution of 19,751 organisms attached to sand-blasted glass plates at *north dock*.

	Barnacles	<i>Acanthodesia tenuis</i>	<i>Electra hastingsae</i>	<i>Bugula neritina</i>	Bivalves	Hydroides sp.	Sponge
0°	1,630	1,881	18	299	3,178	25	25
45°	3,846	1,713	16	194	703	10	26
90°	1,125	344	0	35	246	4	0
135°	1,863	102	0	18	271	2	0
180°	2,029	37	0	1	110	0	0
Total	10,493	4,077	34	547	4,508	41	51

that some may have been mistaken for *A. tenuis*. *Bugula neritina* grew luxuriantly.

The bivalves were principally oysters, although there were some other species present. The results obtained for this group closely resemble those reported by Hopkins (1935) for the Olympia oyster.

Calcareous tubes of an annelid worm, probably belonging to the genus *Hydroides*, and a small white sponge were counted on the various plates. Taxonomic work on both of these organisms is still in progress.

SPECIAL PROBLEMS INVOLVED IN THE STUDY OF BARNACLES

Counts made of the number of cypris larvae and of metamorphosed barnacles have shown no consistent and regular tendency to follow the general pattern observed for other animals reported in this study; namely, that there is a gradual falling off in the numbers of sedentary organisms as the angle of plane surface is increased to 180° (upper horizontal surface). Instead, there is a considerable irregularity in the

relations between population density and the angle of orientation (Tables I, II, III and IV). Values did tend, however, to be elevated at 0 and 45° but depressed at 90°. It was suspected that light might be the primary complicating factor and experiments were therefore designed to test its possible influence on the geotropic response.

In addition to light, other possible variables responsible for the behavior of barnacles under conditions of the experiments were believed to include overcrowding, competition with other organisms, and the influence of primary surface films. In order to test these three variables, very short periods of exposure were tried with special types of plates.

TABLE IV

Numbers of barnacles attached to sand-blasted plates held at various angles

24-Hour (6/5-6/6)				24-Hour (6/11-6/12)			
	Cyp.	Ad.	T.		Cyp.	Ad.	T.
0°	1,370	99	1,469	0°	163	630	793
45°	961	303	1,264	45°	243	854	997
90°	748	181	*929	90°	94	463	557
135°	259	253	512	135°	234	1,057	1,281
180°	129	74	203	180°	253	1,147	1,400
Total	3,467	910	4,377	Total	987	4,151	5,028

5-Day (5/31-6/5)				15-Day (5/31-6/15)			
	Cyp.	Ad.	T.		Cyp.	Ad.	T.
0°	976	2,024	3,000	0°	369	1,261	1,630
45°	554	2,155	2,709	45°	533	3,313	3,846
90°	603	3,285	*3,888	90°	42	1,083	1,125
135°	73	2,443	2,516	135°	25	1,838	1,863
180°	42	2,373	2,415	180°	35	1,994	2,029
Total	2,248	12,280	14,528	Total	1,004	9,489	10,493

* double plates.

Fortunately, the plankton was exceedingly rich in the experimental area during June, 1940, so that the heavy sets of cypris larvae needed for statistically significant analysis could be obtained with very short periods of immersion.

Black, clear and opal glass plates of the type commonly employed as mounting elements in museum jars were selected for these experiments. These plates present smooth surfaces and are useful in testing photic responses without the chemical complications arising when paints are used. All plates measured one and seven-eighths inches by seven and one-quarter inches. Pairs were held in an encircling band of quarter-inch mesh galvanized hardware cloth. Such units offered a total of

approximately 27.2 square inches of surface for each pair of sides. Plates were lowered into Santa Rosa Sound at the end of the *north dock* of the Bureau of Fisheries Laboratory. The mean depth at which they were placed was 8 feet, this being about 3 feet above the bottom. A window weight was used as ballast for each unit. At various intervals plates were removed, washed gently with fresh water and allowed to dry. Counts for both cypris larvae and metamorphosed adults of the barnacle *Balanus cburneus* were made at magnifications of from 6 to 30 times.

While provisional 24-hour experiments with sand-blasted plates (Table IV) had already shown that populations of *B. cburneus* at 180°

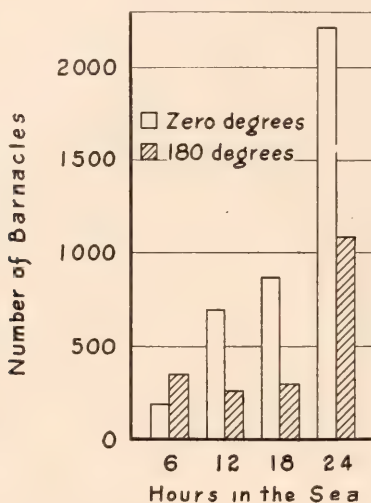


FIG. 1. Barnacle populations on the upper and lower surfaces of plates held in a horizontal position.

were not regularly much lower than those at 0° , these results were confirmed and extended with the use of black plates on whose surface cypris larvae and young adults could be easily counted. An example of this type of test in which 4 sets of plates held in a horizontal position were examined respectively at the end of 6, 12, 18, and 24 hours is shown in Fig. 1. While the increase in numbers of larvae at 0° was regular with time, this was not true for the surface designated as 180° . There were relatively more barnacles on this surface at 6 hours, and by the end of 24 hours the number on the 180° side was about half as great as that found for 0° .

In another series of experiments conducted for 24 hours (Fig. 2) using black, opal and clear plates held both in the horizontal and in the vertical positions, the black plates consistently showed much larger populations. In addition, the variable nature of the response to the geotropic factor was here again confirmed. For all three types of plates the lowest

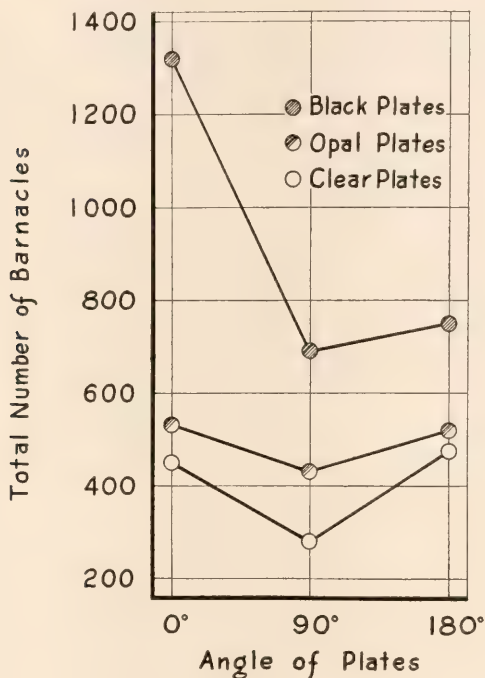


FIG. 2. Orientation of barnacles on black, opal and clear plates held in the horizontal and vertical positions.

counts were found at the 90° angle. The total number of individuals at the three angles were:

Black	2750
Opal	1484
Clear	1204

Table V shows the results of an experiment in which one unit of black and one of clear plates were examined after being held in a horizontal position in the sea for periods of 1, 3, 5, and 7 days. The results show: (a) no significant difference in the population size of the under

TABLE V

Barnacle populations on clear and black plates after exposure for one, three, five and seven days.

Days	Clear			Black		
	0	180	Total	0	180	Total
1	217	287	504	1,514	1,113	2,627
3	851	844	1,695	2,325	2,022	4,347
5	1,006	630	1,636	2,174	2,508	4,682
7	906	863	1,769	1,774	2,303	4,077
	2,980	2,624	5,604	7,787	7,946	15,733

as compared to the upper plane surface; (b) individual and total numbers of barnacles for the various periods were always more than twice as great on black plates; (c) units which remained in the sea 5 and 7 days did not accumulate populations significantly larger than those on the 3-day plates. This last observation suggests that some limiting factor was in operation. If this had been due to the accumulation of silt, its effect should have been most evident on the upper surfaces of plates (180°). A more probable hypothesis is that a slime film which may have formed within the first 5 days became a limiting condition. The fact

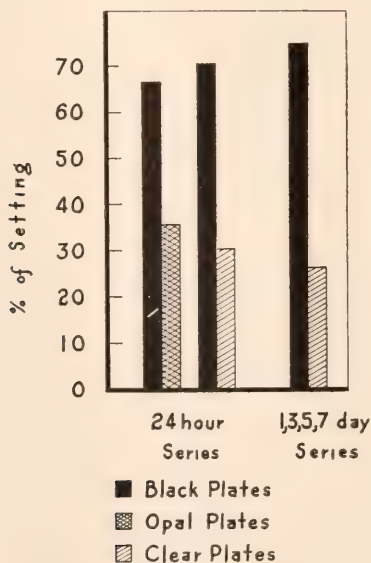


FIG. 3. Percentage of barnacles attached to black versus opal and clear plates.

that a plateau in the population was reached at approximately 1700 individuals on the clear plates, whereas the population on the black plates exposed under identical conditions reached a level of about 4400 individuals, gives evidence that the phenomenon was not due to overcrowding. Bryozoa, bivalves, and sponges were still too few by the end of the seventh day to have been limiting factors. Visscher (1927), quoting Hillen and Angst (1923), has signaled the controversy concerning the significance of primary films and has pointed out the need for further studies of this factor in relation to the attachment of fouling organisms.

The importance of the photic factor in the attachment of barnacles may be seen in Fig. 3, which is a histogram summarizing the percentage of sets on black versus clear and opal plates. In these and other similar experiments, when all other conditions remain constant, twice as many

TABLE VI

Distribution of cypris larvae on pairs of plates offering 27.2 square inches of surface exposed from 9 P.M. to 3 A.M.

Date	Expt. No.	Black			Opal			Clear		
		0°	180°	Total	0°	180°	Total	0°	180°	Total
6/29-30/40	1	62	48	110	42	41	83	29	62	91
"	2	37	13	50	47	53	100	47	53	100
6/30-7/1/40	3	44	39	83	34	65	99	36	72	108
"	4	39	59	98	61	50	111	31	64	95
Totals		182	159	<u>341</u>	184	209	<u>393</u>	143	251	<u>394</u>

barnacles were found attached to black plates as were found on opal or clear plates.

As a method for proving the reality of the photic factor, experiments were conducted on moonless nights between the hours of 9 p.m. and 3 a.m. During these six-hour periods, when visibility was at a minimum, barnacle sets were collected on various types of plates. Population counts were made for two units of plates on two different nights. The total number of organisms on black, opal, or clear plates was remarkably similar (Table VI). Thus it would appear that the outstanding characteristic found in the study of the barnacle, *Balanus eburneus*, that is, the aggregation of notably larger numbers on dark surfaces, must be in the nature of a phototropic response, since the phenomenon does not occur when light is at a minimum.

In his important study on the fouling of ships' bottoms, Visscher (1927) presented evidence showing that barnacles are strongly negative to lights at the time of their attachment. He advocated the quest for substances of light color to serve as an adequate substitute for the red and brown paints which are generally used. The findings of the present study support these conclusions and, in addition, indicate the need for experiments on the effect of illumination in the control of ship fouling.

SUMMARY AND CONCLUSIONS

1. A method is described for studying the attachment of the larvae of sedentary marine organisms to plane surfaces held at various angles to the horizontal. The use of black, opal and clear glass plates to analyze the photic factor is presented.

2. In the curve describing the attachment of six different groups of organisms in relation to the angle at which plates were held, the largest populations always were found at zero degrees (the under surface of plates held in the horizontal position) and the numbers decreased as the angle increased.

3. Planimeter measurements of the colonial bryozoans, *Acanthodesia tenuis* and *Electra hastingseae*, showed further that the area, and therefore the success of the colonies, was likewise a function of the angle at which the plates were held, the greatest growth being at zero degrees.

4. In contrast, the ivory barnacle, *Balanus eburneus*, was not regularly found to orient to the geotropic factor in the same manner as the other sedentary organisms which were encountered.

5. Short-time experiments proved that the initial orientation of the barnacles probably was not limited by other organisms or by silting, although some evidence was found for the view that a primary film may have limited the attachment between the third and fifth day.

6. Under conditions of natural day-night exposure, twice as many barnacles became attached to black as to opal or clear glass plates. In experiments conducted exclusively at night no difference was observed in the size of populations attached to black, opal or clear plates. This suggests that a photic factor is of primary importance in the attachment of the barnacle, *Balanus eburneus*.

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THE DIFFERENTIATION OF GEOGRAPHICAL GROUPS IN *LYMNAEA PALUSTRIS*

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INTRODUCTION

This paper is a first report on a part of the experimental work on individual and group differences in *Lymnaea palustris*, reared under standardized laboratory conditions during the years 1937 to 1940. The actual experiments were conducted jointly by the two authors. The analysis of the data and their presentation in this paper are primarily the work of the first author, who wishes at this point to express full appreciation of the advice and assistance given by Professor Frederick E. Croxton and Mr. Richard H. Brown in connection with the statistical analysis.

The investigation began with a study of *Lymnaea rubella*, indigenous to the Hawaiian Islands, and was later extended to include the local species, *Lymnaea palustris*, for comparison. Two independent collections of *Palustris* were made, one by the second author from a small stream not far from Croton, New York, and the other by the first author from a mill-pond in Newtown, Connecticut. These groups are herein designated as *Lymnaea palustris* Newtown and *Lymnaea palustris* Croton. It soon became clear that the Newtown snails, though morphologically indistinguishable, differed widely from the Croton snails in their physiological processes of growth, fertility, and longevity. The problem was to determine whether these physiological differences were of sufficient magnitude to be significant characteristics of two distinct geographical groups or races, or whether they were to be regarded as the expression of individual variation within one group or population. The problem is of fundamental importance for the analysis of evolution.

An extensive review of the literature is unnecessary here. The problems presented by geographical races are fully discussed by Robson and Richards (1936), Dobzhansky (1937), and by the collaborators of the recent volume "The New Systematics" edited by Huxley (1940); noteworthy chapters in the last-named are those by Huxley (1940), Muller (1940), and Diver (1940). Diver's (1939) general discussion of racial variation must also be mentioned.

For the most part, morphological characters of diverse local groups have been investigated. As representative studies, we may cite those of Rensch (1929) on birds, Gulick (1905) on Achatinellidae, Bartsch (1920) on *Cerions*, Fischer-Piette (1938) on *Patella*, Crampton (1916, 1925, 1932) on *Partula*; Goldschmidt (1934) on *Lymantria*, and Mozley (1935) on *Lymnaea*.

Dobzhansky (1935) has demonstrated that in *Drosophila pseudoobscura* there are two races which are morphologically indistinguishable but physiologically unlike. Working with the same material, Shapiro (1932) and Poulson (1934) have likewise shown that physiological differences between two races exist, while Lancefield's investigation (1929), confirmed by Dobzhansky and Boche (1933), proved that the two races can be distinguished also by the differing forms of the Y chromosome. Recently, Baily (1939) has published an important study on "Physiological Group Differentiation in *Lymnaea columella*."

Our methods of culture of *palustris*, as well as the adopted treatment on the data, differ from those employed by Baily in his work on *columella*. No prior physiological studies on *palustris* are known to us. This species of *Lymnaea* is very different from *columella* and also from *rubella* in its physiological processes of growth and reproduction. It is intermediate between them in the length of its life cycle.

The animals of our experiments were kept in a greenhouse during the winter months in quart jars filled with water to approximately the 500-cc. level. Efforts were made to keep the temperature between 55° and 75° F.; the optimum seemed to be from 60° to 70°. When the temperature grew too warm, toward the end of May, the snails were transferred to cooler laboratories, there to remain until the end of September, when they were returned to the roof greenhouse.

Spring water was used consistently because the animals were adversely affected by the city water. Each jar contained a spray of Cabomba, to provide a substratum for the deposition of eggs, as well as a small amount of finely-sifted earth. The standard food was lettuce of the "Boston" variety, and some of this was always available in the jars.

The individual masses of eggs were removed from the jars soon after they were laid, and were then kept in glass cups of 50-cc. capacity until the young began to hatch. Each cup with its contents was then placed in a standard quart jar where the growing animals passed their early lives in company. Obviously all of the products of a single mass of eggs were kept under identical conditions. When the young snails reached the age of eighty days, they were measured and isolated in the

standard quart jars. Thereafter they were re-measured at eighty-day intervals, and full records of their fertility were secured until they died.

While some of the original wild individuals may have mated before they were collected, the isolated members of the later generations could not have done so. Therefore all of the eggs deposited by the snails of the pedigreed generations were the products of self-fertilization on the part of the animals. As two polar bodies are formed, the possibility of parthenogenesis is excluded. The statistical methods employed in the present study are those which have been developed by Fisher (1938), Mahalanobis (1932), and Snedecor (1934, 1937) for the analysis of variance. When two groups are compared on the basis of measurable characters, the difference between the means may be estimated as significant of a real distinction by the use of the F test. Technically, F is the ratio of the variance between the means to the variance within the separate groups. If the variance between the means is so great as compared with the variance within the separate groups that the variance between the means cannot be regarded as happening by chance, then the hypothesis that the groups have been drawn from the same population must be rejected. It follows then that the two groups show a significant difference, i.e. a difference which is not attributable to chance variation.

In our tables N is the number of individuals in the sample; $\bar{X}$ is the arithmetical mean, and r is the conventional coefficient of correlation; while F is a critical value which, when taken in conjunction with the proper degrees of freedom, can be denoted as significant or not significant by reference to an F table such as that available in "Applied General Statistics" (pp. 878-879) by Croxton and Cowden. The degrees of freedom involved are: between the means, $n_1 = 1$; and within the samples, $n_2 = N_1 - 1 + N_2 - 1$, where N_1 = the number of items in one sample, and N_2 = the number of items in the other sample.

FERTILITY

The term fertility as used in this report refers to the egg-laying activity or productivity of the snails. It has no reference to the viability of the eggs or of the later embryos and growing young.

The original *Lymnaca palustris* constituting the first, or parent generation was collected in October, 1937. It comprised snails that had hatched during the previous spring or early summer, as judged by the observations on the growth and life cycles of later generations. The animals were large enough and old enough to lay soon after they were brought into the laboratory. Only 15 of the 34 Newtown snails and 14 of the 45 Croton snails produced any eggs, whatsoever. In nature these

animals are relatively inactive in winter; we found that no eggs were laid by individuals kept at low temperatures in a cold room.

As shown in Fig. 1, both groups of snails began to lay abundantly during March, and reached the peak of egg production in April. Egg-laying by the Newtown snails then fell off rapidly to a low level in August, after which it was sporadic until December, when the last Newtown snail died at an age of approximately a year and a half. In

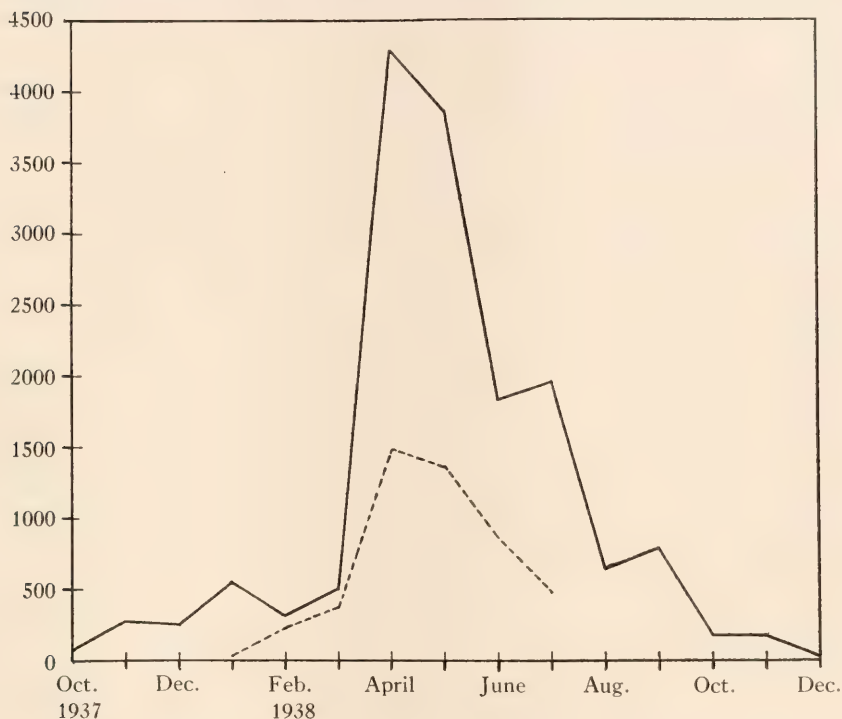


FIG. 1. Egg-laying cycles of the Newtown snails (solid line) and of the Croton snails (broken line) of the parent generation. Y axis—number of eggs produced: 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500. X axis—calendar months: October, 1937 to December, 1938.

contrast, the Croton snails stopped laying quite abruptly in July and they died soon afterward, apparently having reached the end of their normal life cycle, for members of the succeeding generations of this group seldom lived longer than 240 to 320 days. Possibly the heat waves of July hastened their deaths, for it was found later that the Croton snails did not survive extreme hot weather as well as did the Newtown animals.

In comparing the fertilities of the Newtown and Croton groups, several characteristics of reproduction and of the reproductive cycle were

taken into account; namely, the number of egg masses laid, the total number of capsules containing fertile eggs, the number of empty capsules laid, the age of the animal at the onset of fertility, and the length of the fertile period. Table I gives a comparison of the Newtown group with the Croton group in the first, or parent, generation. Each group comprised 15 laying individuals.

The *F* tests show that the Newtown animals were the more productive. They also had a longer fertile period and they laid fewer empty capsules. Further, the Newtown group laid more egg masses than the Croton group, as the means show, but the difference in this respect is not mathematically significant. Colton's statement that the laying of empty capsules comes at the end of the reproductive cycle in *columella*, as a sign

TABLE I

Comparative fertility of the Newtown and Croton snails of the parent generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	15	54.4		
Croton	15	32.0		
			3.332	none
Number of eggs laid				
Newtown	15	1049.80		
Croton	15	320.00		
			9.334	positive
Number of empty capsules				
Newtown	15	5.33		
Croton	15	22.73		
			6.105	positive
Length of fertile period in days				
Newtown	15	122.27		
Croton	15	62.27		
			5.486	positive

of decadence, is borne out by our experience with that species, but in *palustris* this does not seem to be equally true.

Egg production in both groups was ascertained for successive 10-day periods for each snail. In each series the peak of production came in the first or the second 10-day period after the onset of fertility. During the first 20 days of laying, the Croton snails produced 968 eggs in 71 egg masses; the Newtown snails laid 2506 eggs in 91 egg masses. After the peak, production fell off gradually until the 151-160-day period for the Croton group and the 281-290-day period for the Newtown series, and soon afterwards the last survivors died.

The second generation, so-called, comprises the offspring of the original or parent animals. The series described by the figures of Table

II were selected from families whose reproductive cycles were approximately synchronized as to the season of the year. In addition, other calculations were made using random samples of all of the families of this generation and the results are given in Table III. Clearly the group differences that appeared in the first generation were consistently repeated in the second generation, as regards the total number of eggs, the number of empty capsules, and the length of the fertile period. In addition, there was a marked difference between the two series in the age at onset of fertility; here there is no comparison to be drawn with the parent generation, since the ages of the latter were only approximately known.

TABLE II

Comparative fertility of the Newtown and Croton snails of the second generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown.....	24	49.96		
Croton.....	24	19.25		
			12.265	positive
Number of eggs laid				
Newtown.....	24	786.33		
Croton.....	24	132.50		
			11.706	positive
Number of empty capsules				
Newtown.....	24	8.25		
Croton.....	24	34.46		
			8.835	positive
Age at onset of fertility in days				
Newtown.....	19	180.63		
Croton.....	47	97.38		
			122.621	positive
Length of fertile period in days				
Newtown.....	24	98.33		
Croton.....	24	40.79		
			13.430	positive

The possibility of a seasonal variable in the fertility of *palustris* must be taken into account. As already noted (Fig. 1), the members of the parent generation laid few eggs during the autumn and winter and it was not until spring that they began to produce abundantly. Because the reproductive cycle of the Newtown snails differed so much from that of the Croton animals in succeeding generations, it was not possible to demonstrate a seasonal variable in their fertilities. The families compared in Table II came from eggs laid at about the same time of the year, but the Croton snails generally began to lay at earlier ages than the



Newtown snails, and they had a shorter laying span, thus making the exact synchronization of their laying records impossible. The longest laying period of the Croton group was 90 days while that of the Newtown snails was 240 days. Both groups reached the peak of production during the first two 10-day periods of their laying cycles, as in the parent generation. The Croton group actually produced more eggs during the first 20 days, but they ceased to lay much earlier than did the Newtown animals, whose total productivity was much greater in the end.

From our observations, it seems that under laboratory conditions the seasonal rhythm of laying tends to disappear, and that the onset of fertility occurs when the snails reach a certain size, regardless of the time of the year. The significant point is that the characteristic differ-

TABLE III

Comparative fertility of random samples of all Newtown and Croton snails of the second generation

Character	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
Number of egg masses				
Newtown.....	80	39.71		
Croton.....	63	17.22		
			24.731	positive
Number of eggs laid				
Newtown.....	80	506.37		
Croton.....	63	139.36		
			16.192	positive
Number of empty capsules				
Newtown.....	80	7.76		
Croton.....	63	24.41		
			23.151	positive

ences between the Newtown and the Croton groups of the parent generation, as regards the features of the productive cycle, were repeated in the second as well as in the later generations reared in the laboratory.

It was impossible to procure full fertility records for all of the families of the third generation, owing to the abundance of the material. The comparison here made employs the two most productive and long-lived families of the contrasted groups (Table IV). The results of the *F* tests on various aspects of fertility show that the Newtown snails were again more productive than the Croton snails. While the differences are not positively significant, the animals of the former group laid more egg masses throughout a longer period of time, and their ages at the onset of fertility were greater.

Passing to the fourth generation, the Newtown group comprised the relatively large number of 278 snails; the Croton group was made up of 132 individuals belonging to five families, of which only three lived long enough to provide data for complete life cycles. Representative Newtown and Croton families are compared in Table V. Unfortunately, the number of satisfactory Croton layers is small; in fact, the Croton animals proved to be so far inferior to the Newtown snails in their fertility that their breeding had to be discontinued after this generation. The Newtown series, on the other hand, have continued to flourish. They were so weeded out that the stock of later generations has been made up of the descendants of only one of the original snails.

TABLE IV

Comparative fertility of the Newtown and Croton snails of the third generation

Character	N	$\bar{X}$	F	Significance
Number of egg masses				
Newtown	21	24.67		
Croton	12	18.17		
			1.697	none
Number of eggs laid				
Newtown	21	402.81		
Croton	12	238.83		
			4.677	positive
Number of empty capsules				
Newtown	21	2.71		
Croton	12	16.00		
			8.472	positive
Age at onset of fertility in days				
Newtown	21	133.51		
Croton	12	110.25		
			3.218	none
Length of fertile period in days				
Newtown	21	68.30		
Croton	12	52.33		
			1.328	none

The data of Table V are self-sufficient. In this generation it is again clear that the Newtown snails differed from the Croton snails in their actual productivity, and in the same ways as in the earlier series. As the physiological differences in question are exhibited by the two contrasted series throughout four generations, there can be little doubt that the Newtown and Croton groups constitute two geographical races which are presumably unlike in some particulars of their genetic constitutions.

In concluding this section, a partial record is given of the correlations displayed by the several features of fertility with one another and

TABLE V

Comparative fertility of the Newtown and Croton snails of the fourth generation

Character	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
Number of egg masses				
Newtown.....	27	56.15		
Croton.....	7	6.71		
			19.615	positive
Number of eggs laid				
Newtown.....	26	614.46		
Croton.....	7	99.43		
			16.042	positive
Number of empty capsules				
Newtown.....	26	4.61		
Croton.....	7	9.86		
			3.436	none
Age at onset of fertility in days				
Newtown.....	26	180.77		
Croton.....	7	120.43		
			24.471	positive
Length of fertile period in days				
Newtown.....	26	123.96		
Croton.....	7	12.28		
			22.954	positive

with additional characters. The items of Table VI are representative of numerous calculations of data relating to many series.

As would be expected, productivity as indicated in terms of the number of egg masses and of the number of fertile eggs is positively correlated with the length of the fertile period. The total number of eggs laid and the length of the fertile period are correlated with age at death to significant degrees. It is otherwise with the age at onset of fertility, for this feature does not show a definite correlation either with total productivity or with age at death.

TABLE VI

Correlations in three Newtown families of the second generation taken as random samples

Characters	<i>N</i>	<i>r</i>	<i>F</i>	Significance
Number of egg masses and length of fertile period.....	24	.901	94.897	positive
Number of eggs and length of fertile period.....	24	.743	27.107	positive
Number of eggs and age at death...	40	.532	15.016	positive
Length of fertile period and age at death.....	40	.806	70.447	positive
Age at onset of fertility and total productivity.....	40	.258	2.719	none
Age at onset of fertility and age at death.....	40	.177	1.229	none

GROWTH

The main purpose of this section is to compare the Newtown and Croton groups of *palustris* on the basis of the mode of growth and of size at certain stages in the life cycles of the snails. It will appear that differences in these characteristics are quite as definite as in the physiological attributes discussed under the head of fertility.

It will be recalled that the young snails hatched from a single mass of eggs were reared in one and the same nursery jar until they were 80 days old, at which time they were measured and isolated in individual

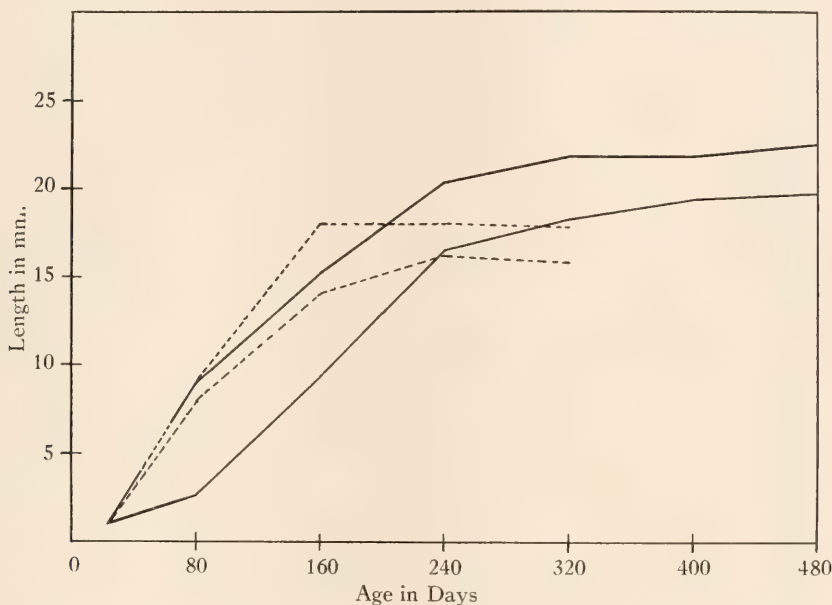


FIG. 2. Growth curves of the fastest-growing and the slowest-growing snails of a typical Newtown family (solid line) and of a typical Croton family (broken line). Y axis—length in mm.: 5, 10, 15, 20, 25. X axis—age in days: 80, 160, 240, 320, 400, 480.

quart jars, thereafter to be measured at 80-day intervals. The total length of the shell was taken as the index of size; measurements of width were also made, but they have not been employed in the present study. In Baily's work on *columella* (1939), the length of the aperture was used as an index of size. Baily measured his animals every five days and thus obtained enough data to plot a logistic curve of growth for each of his two comparable groups. Merrell (1931) investigated the growth of rabbits and plotted a similar logistic curve for each individual and for the whole series of observed animals. Our own meas-

urements are relatively few, but they describe the animals in definite terms at actual successive age-periods in their several cycles. Whatever their limitations, they provide a concrete basis for the determination of group averages and for an estimate of the significance of group differences by the use of the F test. The results are given in Fig. 2 and in Tables VIII to XI.

The length of approximately 1 mm. at about 20 days is taken as the starting point of the curves of growth, even though variations are found at this time and even earlier, just as they are throughout the entire life cycle. During the first hour after a mass of eggs is deposited, water is absorbed by the gelatinous matrix, by the individual egg-capsules, and by the actual eggs themselves, with some degree of enlargement as a consequence. The eggs remain the same in size after the first hour until they begin to divide, about three hours after deposition. The diameter of the eggs of one representative series of *palustris* ranged from 0.108 mm. to 0.126 mm., with an average of 0.115 mm. and a coefficient of variation of 5.36. The early embryos vary considerably even before they reach the time of hatching, and there are also considerable differences between broods as regards the amplitude of variation before emergence. In one Newtown series numbering 30, measured at 13 days, the length ranged from 0.882 mm. to 1.008 mm., the average was 0.952 mm., and the coefficient of variation was 3.69. In another Newtown series of 36, measured at 14 days, the length varied from 0.720 mm. to 1.134 mm., the average was 0.948 mm., and the coefficient of variation was 12.26. It is probable that the larger eggs produce relatively larger embryos. Künkel (1916) has proved this to be the case in *Arion* and in other land pulmonates. But it does not necessarily follow that the individuals which are larger at the time of hatching remain comparatively larger throughout their later lives.

The data of size as indicated by length are given in Table VII for the successive ages of representative broods of *palustris*; the LP1 and LP15 families belonged to the Newtown group while LP63 was a Croton snail. The figures are self-explanatory. They show how the illustrative families differ at the several age-intervals, and how their averages tend to converge in the latter portions of the life cycle. As a matter of detail, in some instances the average size is sometimes less at an older age than it was at an earlier time owing to the death of relatively larger individuals; a case in point is LP15-A at 400 days. In general, absolute variation as indicated by its coefficient is relatively greatest at the 80-day stage and it diminishes as the animals grow older, although there are notable exceptions.

The figures of Table VII and the curves of Fig. 2 show that the contrasted Newtown and Croton groups differ in their modes of growth. The Newtown animals generally increase in size at a slower rate at the outset, but they become comparatively larger at later times and they attain their maximum dimensions at about 320 days. The Croton snails have a shorter life-span as a rule, and they reach their lesser limits of growth by about 240 days—a point that is brought out more definitely in the subsequent section on longevity. The few individuals in every

TABLE VII

Data of size and growth of representative families of the second generation

Age	Family	No.	Arithmetical mean	Standard deviation	Coefficient of variation
<i>days</i>			<i>mm.</i>		
80	LP1-B	38	7.657	1.072	14.00
	LP15-A	11	9.463	2.866	30.29
	LP15-S	23	7.000	1.206	17.23
	LP63-K	74	8.605	2.257	26.23
160	LP1-B	35	10.011	1.463	14.62
	LP15-A	9	13.922	1.127	8.09
	LP15-S	22	21.368	1.317	6.16
	LP63-K	47	17.772	2.071	11.66
240	LP1-B	9	16.622	2.960	17.81
	LP15-A	5	21.000	3.130	14.90
	LP15-S	12	24.525	1.879	7.66
	LP63-K	20	18.525	1.394	7.52
320	LP1-B	5	21.760	2.365	10.87
	LP15-A	3	22.867	3.412	14.92
	LP15-S	7	24.415	1.397	5.72
	LP63-K	4	16.650	0.870	5.22
400	LP1-B	5	22.700	1.227	5.40
	LP15-A	2	21.400	2.545	11.90
	LP15-S	1	24.000		
	LP63-K	0			

family that live beyond the times specified may not increase in size, but if they do, the increments of growth are small.

The contrasted distinctions of the Newtown and Croton groups are consistently the same in all of the material of the second generation. The series described in Table VIII differ somewhat from those of Table VII. The Croton group as a whole is definitely larger in size at 80 days while the members of the Newtown group are the larger at 160 days and 240 days with equal definiteness. An additional calculation

TABLE VIII

Comparative Sizes of the Newtown and Croton snails of the second generation

	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
		<i>mm.</i>		
Size at 80 days				
Newtown.....	38	7.66		
Croton.....	74	8.60		
			5.981	positive
Size at 160 days				
Newtown.....	58	19.97		
Croton.....	47	17.77		
			11.570	positive
Size at 240 days				
Newtown.....	32	21.37		
Croton.....	23	18.41		
			13.865	positive

of 80-day sizes was made for the entire populations belonging to this generation with corroborative results; specifically, the mean size for the Newtown group was 7.150 mm. and the mean of the Croton group was 8.845, with a value for *F* of 15.662 that is certainly positive in significance.

When the third and fourth generations were analyzed (Table IX and Table X), the Newtown and Croton groups showed the same distinctions so far as the conditions at 80 days are concerned. At the 160-day period, the Croton snails were again smaller collectively in the third generation, but they were relatively larger in the fourth generation, although in neither case were the differences significant. In two respects the relations at 240 days were not the same as they were in the second

TABLE IX

Comparative sizes of the Newton and Croton snails of the third generation

	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
		<i>mm.</i>		
Size at 80 days				
Newtown.....	261	6.67		
Croton.....	19	9.94		
			20.461	positive
Size at 160 days				
Newtown.....	251	16.73		
Croton.....	18	15.91		
			1.284	none
Size at 240 days				
Newtown.....	222	18.07		
Croton.....	9	16.58		
			3.691	none

TABLE X

Comparative sizes of the Newtown and Croton snails of the fourth generation

	N	$\bar{X}$ <i>mm.</i>	F	Significance
Size at 80 days				
Newtown.....	132	5.65		
Croton.....	86	7.69		
			36.645	positive
Size at 160 days				
Newtown.....	44	14.69		
Croton.....	43	14.98		
			0.210	none
Size at 240 days				
Newtown.....	38	17.13		
Croton.....	9	19.27		
			9.139	positive

generation. In the first place, the Croton snails did not differ significantly from the Newtown group although they were collectively smaller as before. In the second place, the Croton snails proved to be definitely *larger* at 240 days than the Newtown animals of the same age.

There is no question that the Newtown and Croton groups differ distinctly in mode of growth and in relative size during the earlier months of their life cycles, in all of the pedigreed generations. The circumstances during the later months of life are not so consistent. The reasons why the later generations fail to agree with the second generation are unknown. It may be that unconscious and unavoidable selection is responsible in part, and it may also be that the conditions of laboratory culture exert a modifying influence on the rates of growth in the course of time. However, the Newtown and Croton groups do show differences in certain physiological qualities of growth during their earlier lives, and these differences indicate that the two groups are justly to be regarded as distinguishable geographical races.

TABLE XI

Correlations of size and growth in Newtown snails of the third generation

Characters	N	r	F	Significance
Size at 80 days and size at 320 days.....	87	+.582	43.535	positive
Size at 80 days and increment of size from 80 to 160 days.....	87	-.719	91.075	positive
Size at 320 days and increment of size from 80 to 160 days.....	87	+.216	4.160	positive

Certain correlations of size and mode of growth were determined for some of the series, and the results are given in Table XI. The figures relate to random samples of the Newtown group of the third generation. Size at 80 days and size at 320 days are positively and definitely correlated. In general, the individuals which grew more slowly from the time of hatching to the age of 80 days were the smaller at the last. In other words, the animals tend to retain their respective positions in the graduated scale, from an early age to the virtual end of growth.

The increment of growth from 80 days to 160 days is *negatively* and significantly correlated with size at 80 days. It is clear that the larger snails of the 80-day array grew more slowly during the following interval in question in comparison with the smaller ones, even though the latter generally failed to overtake the former, as the previous correlation has shown. Obviously the form of the curve of growth varies. In some animals it rises rapidly and more precociously to the 80-day stage and thereafter the rate of increase gradually diminishes. In other instances the curve rises more slowly at first, and the transition to a period of more rapid growth falls beyond the 80-day stage. The positive correlation between size at 320 days and the growth increment from 80 days to 160 days is an incidental feature of some interest.

The average age at the onset of fertility is definitely correlated with size at 80 days, with a negative sign. The snails which were relatively larger at 80 days were generally the first to produce eggs. In a Newtown random sample of 12, r was $-.766$ and F was the significant figure of 14.238; in a comparable random sample of 63 Croton snails r was $-.737$ and the value of F , namely, 68.242, was also a significant figure. The two groups show exactly the same correlations even though they differ markedly in their mean ages at the onset of fertility as shown in the previous section.

LONGEVITY

Discussion of the interesting problem of longevity is here confined to the contrast between the Newtown and Croton groups of *palustris*. No data are available for the members of the parent generation because their exact ages when they were brought into the laboratory were unknown; but the following generations have provided abundant information on the length of life of the snails from the date when the eggs were

laid to the time of death. No records were obtained for the animals that died before the age of 80 days, the time of isolation; therefore our calculations are based on random samples of those snails which were alive at the age specified.

From the figures of Table XII it is obvious that the Newtown snails of the 80-day population lived longer than the comparable series of Croton snails. The differences between the two groups are consistent and significant throughout all three generations, and they provide additional evidence that the Newtown and Croton groups constitute different geographical races. A minor point of interest is that erosion of

TABLE XII
Comparative length of life of the Newtown and Croton snails

Generation	N	$\bar{X}$ days	F	Significance
Second				
Newtown	69	245.13		
Croton	71	194.87		
			12.155	positive
Third				
Newtown	23	330.82		
Croton	22	216.77		
			39.567	positive
Fourth				
Newtown	31	332.39		
Croton	23	206.83		
			25.962	positive

the shell—an indication of senescence—often occurred in the Croton snails at or before 240 days, while in the Newtown snails it was seldom observed until after 320 days.

A positive and significant correlation between size at 80 days and age at death is revealed by the records. In a random sample of Newtown snails numbering 53, r was $+.271$ and the value F was 4.200. In general, the snails which grew more rapidly before the 80-day interval lived longer than those with a slower rate of growth in the initial stages. There is no definite correlation between size at 320 days and age of death; in a random sample of 53 individuals, the r was $+.185$ and F was 2.367, which is too small to be significant.

STERILITY

The occurrence in *palustris* of numerous individuals that did not reproduce raises questions of considerable interest. The treatment of the problems of their sterility encounters certain difficulties, partly because the age when the contrasted fertile animals begin to produce eggs is widely variable.

In the case of the original or parent generation, 19 out of 34 Newtown snails (57.3 per cent) died without laying any eggs although they grew to full size, while 31 of the 45 Croton snails (68.9 per cent) were also sterile. Although their proportionate numbers varied from family to family, some of the members of the later generations failed to produce eggs although they lived well beyond the *average* age of onset of fertility of their respective groups. In the following discussion, however, only those individuals which lived beyond the *last* onset among their associates in the group, without laying eggs, are treated as sterile.

When the fertile and the sterile series were compared on the basis of relative size at successive age intervals (Table XIII and Table XIV), the members of the productive group were uniformly the larger at all periods of the life cycle. The Newtown and Croton series displayed no differences in these relations. But the two geographical series are unlike as regards longevity (Table XV). The general age at death of the fertile animals was definitely greater in the case of the Newtown animals; in the case of the Croton series, however, the difference between the fertile and sterile components was not statistically significant, although here again the animals which produced eggs were the larger collectively. Hence sterility is certainly associated with smaller size and shorter life in the *palustris* from the Newtown area, while it is associated with smaller size, but not with shorter life, in the Croton group.

Our animals were isolated at the age of 80 days before they reproduced and any eggs which were subsequently produced were certainly the products of self-fertilization. It is possible that some of the sterile individuals might have been fertile if they had been given an opportunity to mate, but there is no way to determine whether this might have been the case.

Self-fertilization in itself does not seem to have a detrimental effect upon the viability of the young or upon the productivity of fertile individuals in *palustris*; in fact, members of the fifth, sixth and seventh generations were even more prolific than those of the second generation. Crabb (1927) states that individuals of *Lymnaea stagnalis appressa* "reared in strict isolation reproduce as abundantly as do those in mass cultures." In the work of Colton and Pennypacker (1934) on *columella*,

TABLE XIII

Comparison of size of the fertile and sterile Newtown snails of the second generation

	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
		<i>mm.</i>		
Size at 80 days				
fertile.....	56	6.90		
sterile.....	47	5.19		
			13.839	positive
Size at 160 days				
fertile.....	56	17.73		
sterile.....	37	16.10		
			8.640	positive
Size at 240 days				
fertile.....	173	18.53		
sterile.....	28	15.21		
			17.554	positive

93 consecutive generations were reared between 1911 and 1934 with self-fertilization throughout this period of years, and at the end the animals were hardy and prolific. Incidentally, sterile individuals are far more infrequent in *columella* than they are in *palustris*. Boycott, Diver, Garstang and Turner (1930) found that in *Lymnaca peregra* the animals would mate and cross-fertilize if they were given an opportunity to do so, but if they were reared in isolation they would produce fertile eggs equally well although at somewhat older ages.

But the point of greatest interest is that in our material of *palustris* absolute sterility is associated with smaller size. This suggests that the

TABLE XIV

Comparison of size of fertile and sterile Croton snails

	<i>N</i>	$\bar{X}$	<i>F</i>	Significance
		<i>mm.</i>		
Size at 80 days				
fertile.....	63	10.09		
sterile.....	28	7.73		
			38.333	positive
Size at 160 days				
fertile.....	51	17.57		
sterile.....	10	14.51		
			9.045	positive
Size at 240 days				
fertile.....	40	18.09		
sterile.....	13	15.67		
			22.559	positive

failure to lay eggs is due to retarded development or to an actual constitutional incapacity and not to the lack of an opportunity to mate. It is possible that real recessive defects involving the reproductive apparatus and its functions may exist, and that inbreeding might therefore increase the relative numbers of infertile individuals.

SUMMARY

Variation in *Lymnaca palustris* is exhibited in fertility, rate of growth, size, and longevity. Individual, family and group differences recur in successive generations in spite of the essential uniformity of the laboratory conditions of culture; hence their genetic causation seems probable.

Sample populations collected from two different localities and habitats were analyzed by the *F* test for significant differences in fertility,

TABLE XV

Comparison of age at death of the fertile and sterile snails of the second generation

	<i>N</i>	$\bar{X}$ <i>days</i>	<i>F</i>	Significance
Newtown group				
fertile.....	157	272.94		
sterile.....	34	223.29		
			9.991	positive
Croton group				
fertile.....	62	209.37		
sterile.....	24	196.04		
			1.053	none

growth, and longevity. The results demonstrated the reality of definite differences in all three categories.

The study of group differences was continued throughout three consecutive offspring generations, and in each of these the differences demonstrated in the parent generation were found to be repeated. The two local populations are therefore to be regarded as different geographical races.

Significant and positive correlations were found between the total number of eggs laid and the age at death, between the number of eggs laid and the length of the fertile period, and between the length of the fertile period and age at death.

Extreme types of slow-growing and fast-growing individuals were found in every group with intermediates of great variety. When arranged in order of size at 80 days, the snails exhibited a tendency to

keep their respective places throughout the rest of the life cycle, as indicated by the positive correlation between size at 80 days and size at 320 days. The rate of growth between 80 days and 160 days is inversely correlated with size at 80 days. The critical period in the growth curve occurs relatively early in the life cycle when the individual mode of growth appears to be established.

The onset of fertility is negatively correlated as to time with size at 80 days. The correlation in question is the same in the two local series, despite their demonstrated differences in size at 80 days and in age at the onset of fertility.

Some of the individuals in each population were sterile. When a study was made to ascertain whether such animals differed significantly from the fertile snails, the *F* test showed that they were collectively smaller than the productive individuals and that their lives were shorter.

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SOME OBSERVATIONS REGARDING THE NUCLEOLUS AND CYTOPLASM IN LIVING MARINE EGGS

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Laboratory, Woods Hole, Mass.)

An attempt to determine further the chemical nature of nucleoli, which arose in connection with a survey of the nucleolus, now in press, has led to the following observations. Marine eggs were chosen as most suitable for the purpose. In fresh eggs of *Asterias forbesii* mounted in sea water under an immersion lens the nucleolus is relatively large and generally appears to contain two immiscible substances, the central droplet appearing faintly yellowish and more highly refractive than the surrounding colorless part. The central part, or nucleolus, is sometimes in several droplets. The peripheral part is generally uniform, but in some nucleoli has a granular, in others a reticulate appearance. In some nuclei these two parts become separated as two unequal spheres, the nucleolus being the smaller in volume.

In eggs of *Macrura solidissima* the nucleolus similarly consists of two unequal parts, but the nucleolus instead of being concentrically placed is generally eccentric, touching one side of the nucleolar surface from the inside. It may contain one or more distinguishable globules or vacuoles. Not infrequently there are two nucleoli of unequal size, the larger containing as usual a more or less spherical eccentrically placed nucleolus.

It is now known that the nucleolus or plasmosome normally contains no chromatin, but the two substances here observed evidently correspond to the basophilic and oxyphilic substances so frequently described in nucleoli by animal histologists and by cytologists in animal eggs (see Wilson's "Cell," third edition, p. 270).

Attempts to obtain tests for lipoids from the nucleolus gave negative results. When fresh *Asterias* eggs are treated with Sudan III (0.1 per cent in 95 per cent alcohol) the egg cytoplasm stains yellow and the reagent is precipitated in orange-colored masses in the sea water around the eggs. While this confirms the presence of lipoids in the cytoplasm, the nucleus remains clear and there is no certainty that the color of the faintly yellowish nucleolus is increased. Eggs which had

been killed by heating for five minutes on a water bath at 60° C. give the same result. When fresh *Mactra* eggs were treated with Sudan III the cytoplasm became pale yellow and small yellow aggregates formed just under the egg membrane, but no effect could be detected in the nucleolus. Similarly, when eggs of *Mactra* and *Asterias* were treated with 2 per cent osmic acid, no blackening effect on the nucleolus could be certainly observed, although the cytoplasm of *Mactra* became muddy and that of *Asterias* became yellowish with a delicate network of black. When fresh eggs of *Mactra* are dried on the slide by flaming and then treated with Scharlach R in alcohol the cytoplasm stains pink, showing the presence of fats, but the nuclei and nucleoli remain clear. When fresh *Mactra* eggs are mounted in chloroform on a slide, they become so granular that the nucleolus cannot be observed. If eggs are flamed and dried, then chloroform added, the nucleolus remains unchanged, although this reagent would be expected to dissolve any fats.

These results seem to negative the view that the nucleolus, at any rate of animal eggs, commonly contains ordinary lipoids.

Ruthenium red is regarded as a characteristic reagent for volutin in animals and pectin in plants. When a 0.2 per cent aqueous solution was added to fresh *Mactra* eggs the cytoplasm became more or less pink, which may have been merely an *intra vitam* stain, but the nucleolus remained uncolored.

Claude (1940) has found that by centrifuging normal and tumor cells, particles are separated—probably mitochondria—which contain phospholipids.

To test the possible presence of phospholipids in the nucleoli of animal eggs, about 15 cc. of Feulgen solution was added to four drops of *Asterias* eggs in sea water. Without any hydrolysis, the egg cytoplasm begins to be colored in less than a minute, the characteristic magenta color gradually getting deeper while the nucleus remains uncolored and the nucleolus in many eggs becomes invisible, probably dissolving. On standing, the egg cytoplasm becomes brilliant magenta, the nuclei remaining clear. When a mass of the polygonal ovarian eggs is similarly treated with Feulgen, the color appears at once and is even more intense, indicating a greater concentration of some substance containing a free aldehyde group. The peripheral free eggs in such a mass are at first uncolored. Then the egg membrane appears magenta. When 5–6 cc. of Feulgen were added to three drops of concentrated eggs in distilled water, the nucleolus was dissolved at once and the cytoplasm shortly began to take on the characteristic color. About half the eggs were cytolized by the fresh water.

The appearance of the magenta color in unhydrolyzed cells indicates the presence in the cytoplasm of substances having a free aldehyde group. Such a class of lipoidal substances has been found by Feulgen and Voit (1924) in the cytoplasm of animal tissues and of an intestinal ciliate. The substance plasmalogen is insoluble in water, but with acids or sublimate splits off an aldehyde, plasmal, which gives the violet coloration with Schiff's reagent. The rate of coloration in any case will then depend on the rate of change from plasmalogen to plasmal, which may depend in turn on the fineness of colloidal dispersion of the plasmalogen in the cell.

Plasmal was later found (Feulgen and Bersin, 1939) to be a mixture of higher aldehydes of the known fatty acids, was extracted from horse flesh with alcohol, and a formula for plasmalogenic acid was obtained. This nitrogen-free acid resembles the phosphatid acids obtained from cabbage and spinach by Chibnall and Channon (1927). It appears that the substances in the cytoplasm of the starfish and other marine eggs, which give this strong magenta coloration with Feulgen, without hydrolysis, are aldehydes belonging to the class of acetalphosphatids. The classical brain phosphatids were found to contain as much as 20 per cent of acetalphosphatid. Whether they are contained in "mitochondria" cannot be stated. However, observations of *Chaetopterus* eggs treated with Feulgen under a 2 mm. immersion lens show that many of the granules in the cytoplasm take the magenta color. In crushed eggs some granules are deep magenta, some pale, some uncolored. There is also a diffuse pink color in the cytoplasm. Some of the magenta granules are of maximum size, some very minute, and the smallest visible granules take the color most intensely while some granules of all sizes remain uncolored.

Although the cytoplasm of the *Chaetopterus* egg is colorless, in Feulgen these eggs take the color much more slowly than *Asterias* eggs and only show it definitely after nearly an hour. The area of the maturation spindle remains clear. By the next day the cytoplasm is intensely purple. The muscle fibres in the fans of *Chaetopterus* are colored in the same way, the surrounding tissue remaining unaffected. *Macrura* eggs in Feulgen begin to show the color in about ten minutes and the cytoplasm is definitely colored in about forty-five minutes. *Arbacia* eggs also develop the magenta color slowly, perhaps because it is obscured by the red pigment already present in the cytoplasm, but it finally appears in its full intensity.

The plasmalogens were found by Feulgen and Voit to be insoluble in water, soluble in alcohol and organic solvents. To test these proper-

ties, eggs of *Arbacia* and of *Chaetopterus* were killed by being heated in a water bath, then washed in sea-water, fifteen changes being made in about fifty-four hours. If Feulgen is then added to these *Arbacia* eggs, they shortly begin to change to orange color; in half an hour many of the eggs are magenta and by next morning all show this color. In *Chaetopterus* eggs, after washing, the cytoplasm is granular but none seem cytolized. When Feulgen is added, the cytoplasm of some eggs begins to show a faint rosy tint after five minutes and most of them are full magenta in half an hour, but the color is less intense than in unwashed eggs. A few eggs remained quite colorless. While these plasmalogens are then relatively insoluble in water, there is evidence that a small amount has been washed out, but the sea water from washed eggs, when tested with Feulgen, gave negative results.

Fresh *Macra* eggs were left two days in 95 per cent alcohol. Ordinary alcohol quickly turns pink when Feulgen is added to it, because of the aldehyde present; and it was not evident that the alcohol from these eggs changed color more quickly or more deeply. But the eggs, when tested with Feulgen, were only faint pink after five minutes; after an hour the cytoplasm was pale pink and the nucleus had changed its appearance, being pale pink, the nucleolus uncolored. From this it appears that most of the plasmalogens have been extracted from the cytoplasm by alcohol. *Macra* eggs were flamed on the slide, then placed in 1 per cent sublimate for three minutes, then washed in distilled water and mounted in Feulgen. In a few minutes the cytoplasm is pink and after an hour the eggs remain rather pale pink. If, as stated by Feulgen and Voit, sublimate accelerates the separation of the aldehyde plasmal from plasmalogen, a more intense coloration would be expected.

From these preliminary experiments it can be stated that the aldehyde substances in the cytoplasm of invertebrate animal eggs agree in many respects with those lipoidal substances found to be of widespread occurrence in animal tissues, but they may differ in certain particulars. The absence of phospholipids or other lipoids from the nucleoli seems to be definitely shown. Attempts with *Macra* eggs to throw out the nucleolus, in order to make mass tests, were unsuccessful at a centrifuge speed of about 70,000 g.

It has already been mentioned that in eggs of *Asterias* and *Macra* the nucleolus consists of two unequal parts, one enclosed within the other. These are soluble in tap water; but if water is added slowly while the eggs are under observation with an immersion lens, it can be seen that they show marked differential solubility, the internal "nucleolinus" generally remaining long after the outer part has disappeared.

During this process the two parts may become separated. Gersch (1940) has found that in frog's eggs when the nuclei are separated out the nucleoli are soluble in distilled water.

Egg cells and oogonia of *Fucus* in sea water are negative to Feulgen at ordinary temperatures, showing no change in color after an hour and more. When exposed to air, after Feulgen treatment the thallus turns purple. At a higher temperature both egg cells and the colorless strands in the medulla of the thallus become pink or magenta and dark magenta bodies appear in the cytoplasm of these medullary filaments. It has not been possible to make any further experiments on this subject.

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THE OSMOTIC PROPERTIES OF THE NUCLEUS

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INTRODUCTION

In spite of the fact that extensive investigations have been carried out upon the osmotic behavior of cells and tissues, comparatively little attention has been devoted to the study of the osmotic properties of the nucleus. There is, however, considerable evidence in the literature that the nuclear membrane is semi-permeable. Thus Shinke (1937) was able to demonstrate osmotic swelling and shrinking for the nuclei of perhaps twenty different species of plant cells. He found too that the nuclei of the intestinal epithelium of *Triturus pyrrhogaster*, the nuclei of the salivary gland cells of *Chironomus dorsalis* and the nuclei of the first spermatocytes of *Tryxalis nasuta* react osmotically. Reversible swelling and shrinking of the nuclei of the salivary gland cells of dipterous larvae have been observed by Buck and Boche (1938). Olivo (1938) reported that the nuclei of myocardial cells swell in distilled water. Küster (1932) produced "Niederschlagsmembranen" upon the surface of nuclei of various plant cells by treatment with injurious agents, and states that these membranes are semi-permeable. Further references to the osmotic properties of the nucleus under pathological conditions will be found in Küster's paper. Finally, Beck and Shapiro (1936), studying the permeability to water of the starfish egg and its germinal vesicle, made certain conjectures concerning the relative amounts of osmotically inactive substance in cell and nucleus.

The cursory observations reported above are supplemented by two papers of a more rigorous, quantitative nature. One of these consists of the extensive series of investigations conducted many years ago by Hamburger (1904). He not only made semi-quantitative determinations of the degree of swelling and shrinking of the nuclei of different types of gut epithelia in anisotonic NaCl solutions, but even succeeded in estimating the amount of osmotically inert material ("Gerüstsubstanz," Hamburger; "colloid substance," Hardy) in the frog spermatozoön. In addition he noted that isolated nuclei can react osmotically.

Probably the most extensive data compiled on the osmotic behavior of the nucleus are those of Skowron and Skowron (1926) for the germinal vesicle of the egg of *Sphaerechinus granularis*. In this work the diameters of the cell and germinal vesicle were measured in different dextrose solutions whose freezing-point depressions were known. They found that the cell and nucleus are impermeable to dextrose and that both swell in hypotonic solutions. They state further that, although the cell as a whole shrinks in a hypertonic dextrose solution ($\Delta T = -2.57^{\circ}$ C.), the nucleus undergoes no diminution in volume. This latter observation can only be interpreted as meaning that the germinal vesicle of the *Sphaerechinus* egg does not behave osmotically; a conclusion at variance with the observations to be presented here on the osmotic properties of the germinal vesicle of the immature egg of *Arbacia punctulata* and that of the mature, unfertilized egg of *Nereis limbata*.

MATERIALS AND METHODS

The immature eggs of *Arbacia punctulata* and the mature, unfertilized eggs of *Nereis limbata*—two marine invertebrates—were used in these experiments. These were chosen for study because the nucleus (germinal vesicle) of each is large and spherical, and its volume can easily be determined.

Immature *Arbacia* eggs were obtained as follows. The ovaries were removed from the test to a dish of sea water, whereupon the mature eggs were extruded. A fine pipette was then inserted into the "collapsed" ovary and many immature eggs, of various sizes and in different stages of development, were removed. Since these eggs are aspherical in the ovary and only slowly round up in sea water, it was found advisable to allow them to remain in sea water for about ten minutes before starting an experiment.

The immature eggs were transferred to a glass chamber of approximately 25 cc. capacity and a suitable cell was chosen for microscopical study. Measurements of the diameters of the cell and its germinal vesicle were made using one of two methods: projection by means of a camera lucida, or photographically. The photographic method will be described in detail in a future paper dealing with the permeability of the nucleus to water. Measurements were consistent to 0.4μ with either technique. No attempt was made to maintain a constant temperature.

After determining the diameters of the cell and its nucleus in 100 per cent sea water, the superfluous sea water was removed with a micropipette until less than 0.5 cc. remained directly under the water

immersion lens (40 $\times$). The experimental solution was now carefully added and the equilibrium diameters of the cell and its nucleus determined 30 minutes later. Then the experimental solution was removed, sea water added, and the measurements once more carried out upon the attainment of equilibrium.

In general the same procedure was followed in the experiments with the *Nereis* eggs. These were obtained by snipping the body of the worm in a finger bowl containing sea water, and were then arranged for microscopical study as described for the *Arbacia* material. In this case, however, only the nuclei were measured. Moreover, instead of carrying out a complete experiment, involving reversible swelling and shrinking of a single nucleus, the nuclei of 50 or more cells were measured in any one experiment. In order to reduce the biological variations, only the eggs from one animal were used for an experiment.

The solutions were prepared by appropriately diluting sea water with distilled water (hypotonic solutions), and by concentrating sea water by forced evaporation to obtain hypertonic solutions whose freezing-point depressions had been previously determined (Churney, 1940).

In addition to the experimental techniques already described, another series of experiments was carried out on the *Nereis* nuclei. These were designed to determine whether or not the final volumes reached by the nuclei at equilibrium in anisotonic solutions are dependent upon the path followed. The experiment was very simple. Eggs from a single female were divided into two sets and about 50 nuclei measured in each. One group was then transferred to 80 per cent sea water; the other to 60 per cent sea water. A half-hour later 50 nuclei in the 80 per cent sea water solution were measured. The 80 per cent sea water was then removed from the chamber and a 60 per cent solution substituted. Following attainment of equilibrium 30 minutes later, the diameters of 50 nuclei were measured in each of the dishes containing 60 per cent sea water. The same experimental procedure was performed using 70 and 50 per cent sea water.

RESULTS AND DISCUSSION

In accordance with the treatment of the subject by Lucké and McCutcheon (1932), two conditions must be satisfied for a cell or nucleus to behave as an osmometer. First it must have a semi-permeable membrane. This may be defined as one which permits the passage of the solvent, water, but excludes the solutes, mainly salts. To test the assumption of a semi-permeable membrane, data have been compiled showing the volumes attained by cells and nuclei

upon return to sea water after previous immersion in hypertonic or hypotonic solutions. If the solutes do not penetrate, the initial volume of the cell or nucleus, V_0 , in sea water will be equal to the equilibrium volume, V_0' , assumed upon return to sea water after immersion in an anisotonic solution. Discrepancies serve as a measure of the deviation of the membrane from semi-permeability. In case of equal and reversible exchange of solutes during swelling and shrinking this relationship would be camouflaged.

In the second place the osmotic behavior of the cell must follow the Boyle-van't Hoff law. This principle states that at equilibrium the product of the cell volume by the osmotic pressure of the external solution is a constant for all concentrations; or,

$$PV = K.$$

At equilibrium the osmotic pressure within the cell is considered equal to that of the external solution. Since the cells are always in equilibrium with 100 per cent sea water, P_0V_0 , the equation takes the form

$$P_0V_0 = P_1V_1,$$

where P_1V_1 represents the pressure-volume product of the cells at equilibrium in anisotonic solutions.

As pointed out many years ago by Hamburger (1904), and extensively investigated by Lucké and McCutcheon (1932), the Boyle-van't Hoff law holds only for ideal solutions. This means that, for the cell and nucleus to obey this rule perfectly, the cell contents would have to be infinitely dilute and contain no osmotically inactive material. To correct for the "non-solvent volume," Lucké and McCutcheon introduced a factor " b ," which is independent of the external osmotic pressure. The equation then becomes

$$P_0(V_0 - b) = P_1(V_1 - b).$$

Data for the immature *Arbacia* egg are presented in Table I. Agreement with the Boyle-van't Hoff law is determined by equating the values for P_0V_0 with those for P_1V_1 (columns 3 and 4 respectively) for any one relative pressure (column 2). The deviations are given in column 5. The results indicate: (1) that there is good adherence to the Boyle-van't Hoff law in the case of solutions whose osmotic pressure is close to that of sea water (1.13, 0.9, 0.8 times); and (2) that as one approaches greater dilutions, the deviations increase considerably and show a decided drift. The negative signs in column 5 signify that, in the case of the hypotonic solutions, the cells do not swell to the required volumes. It should be pointed out that there is some evidence that 50 per cent sea water is injurious. In the other

TABLE I
Immature eggs of *Arbacia punctulata*

Number of Cells	Relative Pressures	P_0V_0	P_1V_1	Percentage of Deviation
13	1.13	189.9	194.9	2.6
21	0.9	191.0	185.9	-2.7
26	0.8	198.8	190.8	-4.0
33	0.7	217.7	198.6	-8.8
40	0.6	208.8	184.8	-11.5
34	0.5	214.0	178.2	-16.7

direction it was found that a hypertonic solution equivalent to 131.0 per cent sea water represents the limiting concentration, because of the fact that in higher concentrations the cells form blebs and are no longer spherical.

In Table II is presented a similar set of data for the egg of *Sphaerechinus granularis* taken from the data of Skowron and Skowron (1926). Columns 1, 2, 3, and 6 are arranged from their various tables; columns 4 and 5 have been compiled by the writer. The freezing-point depression of the sea water at Naples is given as -2.28°C ., so that " $\Delta T \cdot V_1$ " = 74.51 (column 4) is really P_0V_0 . The remaining figures in this column are P_1V_1 .¹ The agreement between the values for P_1V_1 and that for P_0V_0 (= 74.51) is fair except in the case of the solution whose freezing-point depression is -1.26°C ., which is equivalent approximately to 55 per cent sea water. In the case of the most hypotonic solution ($\Delta T = -0.97^\circ\text{C}$.), injury is manifested by loss of semi-permeability and by cytolysis. That the data in general are in harmony with the Boyle-van't Hoff law can be seen from their "Diagramme 1" (not reproduced), in which "Masse" is plotted as the

¹ The arbitrary figures (" cm^3 ") for the volumes given by Skowron and Skowron may readily be converted into cubic micra by noting that, "Nous avons tracé les contours des oeufs à l'aide d'un appareil à dessiner à un grossissement de 390 fois, à la hauteur de la platine du microscope." Thus for a cell whose volume is 32.683 cm^3 , we have

$$V = \frac{32.683 \times 10^{12}}{(390)^3} = \frac{32.683 \times 10^{12}}{5.9319 \times 10^7} = 550 \times 10^3 \mu^3$$

The diameter of such a cell will be given by,

$$D = \sqrt[3]{\frac{6V}{\pi}} = 101.6\mu.$$

Likewise the volume of the nucleus of this cell is given as 4.268 cm^3 (Table IV); its volume in cubic micra is

$$V = \frac{4.268 \times 10^{11}}{5.9319 \times 10^7} = 70.3 \times 10^3 \mu^3,$$

and its diameter, 51.2 μ .

TABLE II
Eggs of *Sphaerechinus granularis*
(after Skowron and Skowron, 1926)

Number of Cells	Pressures ΔT	Equilibrium Volumes "cm. ³ "	$\Delta T \cdot V_1$	Percentage of Deviation	Remarks
14	2.28	32.683	74.51		
7	1.73	41.293	71.43	-4.1	
11	1.70	42.673	72.54	-2.6	
12	1.26	50.871	64.10	-14.0	
10	1.08	67.448	72.85	-2.2	cytolysis
10	0.97	103.735	100.63	35.0	cytolysis

ordinate and " Δ " as the abscissa. The resulting curve is a fair rectangular hyperbola, as is required if the equation $P \cdot V = K$ is satisfied. In addition they report that the cell as a whole shrinks in hypertonic solutions.

A discussion of the "non-solvent volume," b , will be reserved until after the data for the nucleus have been presented.

In Table III are arranged the data for the osmotic properties of the germinal vesicle of the immature *Arbacia* egg. A test of the semi-permeability of the nuclear membrane may be made by comparing the values of $P_0 V_0$ with those for $P_0' V_0'$ (columns 7 and 8 respectively) for any one relative pressure (column 2). The deviations in percentage are given in column 9. It is a matter of regret that more nuclei were not measured, yet it appears certain that the nuclear membrane is truly semi-permeable.

Comparing the deviations of the values for $P_0 V_0$ with those for $P_1 V_1$ (column 5) one arrives at the following conclusions: (1) adherence to the Boyle-van't Hoff law is good; (2) as in the case of the figures

TABLE III
Germinal vesicles of *Arbacia punctulata*

Number of Nuclei	Relative Pressures	$P_0 V_0$	$P_1 V_1$	Percentage of Deviation	Number of Nuclei	$P_0 V_0$	$P_0' V_0'$	Percentage of Deviation
6	1.13	30.8	28.1	8.8	3	34.0	32.0	5.9
17	0.9	20.5	20.4	-0.5	10	20.5	20.4	-0.5
46	0.8	27.5	27.5	0.0	13	28.2	27.9	-1.1
51	0.7	27.5	26.8	-2.5	24	28.1	28.7	2.1
53	0.6	28.0	26.4	-5.7	19	27.5	27.5	0.0
48	0.5	28.8	25.4	-11.8	8	28.0	27.9	-0.4

for the cell as a whole, there is better agreement for solutions whose osmotic pressure is close to that of sea water; (3) there is a marked drift in the deviations with increasing dilutions; and (4) the percentage deviations are considerably smaller for the nucleus than for the ce

as a whole. One is strongly tempted to draw the conclusion that the nucleus is a better osmometer than the cell.

For comparison with the results cited for the *Arbacia* nucleus, Table IV, which deals with experiments on the osmotic properties of

TABLE IV
Nuclei of *Sphaerechinus granularis*
(after Skowron and Skowron, 1926)

Number of Nuclei	Pressures ΔT	Equilibrium Volumes "cm. ³ "	$\Delta T \cdot V_1$	Percentage of Deviation
14	2.28	4.268	9.74	
7	1.73	6.254	10.81	10.9
11	1.70	6.954	11.82	21.2
12	1.26	7.553	9.51	-2.4
10	1.08	10.384	11.21	15.1
10	0.97	6.018	5.84	-40.1

the germinal vesicle of the *Sphaerechinus* egg, has been prepared from the data of Skowron and Skowron. Columns 1, 2, and 3 have been taken directly from their tables and columns 4 and 5 compiled by the writer. The average of the equilibrium volumes in sea water ($P_0 V_0$) is 9.74. All the other figures in column 4 correspond to $P_1 V_1$. In this case the figures of column 5 indicate considerable deviation from obedience to the Boyle-van't Hoff law and contrast sharply with the results just presented for the germinal vesicle of the *Arbacia* egg

TABLE V
Germinal vesicles of *Nereis limbata*

Relative Pressure	Number of Nuclei, V_0	Number of Nuclei, V_1	$P_0 V_0$	$P_1 V_1$	Percentage of Deviation	Number of Nuclei, V_0'	$P_0' V_0'$	Percentage of Deviation
0.9	56	64	98.37	96.34	-2.06	27	95.90	-2.51
0.9	73	61	103.33	101.83	-1.45	60	107.62	4.15
0.8	51	43	101.14	94.21	-6.85	38	104.33	3.15
0.7	53	56	100.67	84.32	-16.24			
0.7	57	58	98.53	87.86	-10.83	20	95.77	-2.80
0.6	27	49	127.47	102.24	-19.78	51	123.82	-2.86
0.5	49	44	108.99	77.56	-28.83	35	116.63	7.01

(Table III). It may be, of course, that the germinal vesicle of the *Sphaerechinus* egg does not obey the Boyle-van't Hoff law. Indeed, their statement that the nucleus does not shrink in hypertonic solutions points definitely to this conclusion. However, it seems likely, as the investigators themselves admit, that accurate results cannot be obtained for the nucleus by their method. It would certainly be de-

sirable to have more information concerning the accuracy of their technique.

Evidence that the nucleus is an osmometer has been extended to the case of the germinal vesicle of the *Nereis* egg (Tables V and VI). The criterion of semi-permeability may be applied by comparing the values of P_0V_0 (initial volumes) with those for $P'_0V'_0$ (volumes assumed upon return to 100 per cent sea water after a swelling experiment). The data are presented in Table V, columns 4 and 8 respectively. All the deviations are greater than those for the *Arbacia* nuclei. The values fluctuate more or less at random about zero and indicate that the nuclear membrane is probably semi-permeable. The entry for 50 per cent sea water is difficult to understand, since its algebraic sign is the opposite of what one would expect if salts were being lost during the swelling process.

TABLE VI
Germinal vesicles of *Nereis limbata*

P_0V_0	P_1V_1 in 80% Sea Water	Percentage of Deviation	$P'_1V'_1$ in 60% Sea Water	Percentage of Deviation
A. 90.17 B. 91.71	90.34	0.19	78.67 81.52	-12.75 -11.11
P_0V_0	P_1V_1 in 70% Sea Water	Percentage of Deviation	$P'_1V'_1$ in 50% Sea Water	Percentage of Deviation
A. 96.44 B. 95.60	81.95	-15.02	68.15 71.14	-29.33 -25.58

In order to gain more information on this point, another sort of experimental attack was attempted. The procedure, already described under the heading "Materials and Methods," consisted in comparing the averaged volumes of two sets of nuclei brought by different paths into equilibrium with any given solution. In one case the nuclei were transferred directly from 100 per cent sea water to a diluted medium; in the other, only after a previous immersion in a solution of some intermediate concentration.

The reasoning underlying this experiment is based on the assumption that leakage is a function, among other things, of the concentration gradient. That is, the degree to which salts will tend to pass out of the nucleus will depend upon the rate of swelling. It may be expected, then, that the final volumes attained by the nuclei at equilibrium in any anisotonic solution will depend upon the path taken.

The results of these experiments show definitely, however, that the nuclei attain their final volumes quite independently of the path

traversed. Thus, for the series in 80 and 60 per cent sea water, the deviation from the volumes demanded by the Boyle-van't Hoff law is 11.11 per cent in the case of the nuclei transferred directly to 60 per cent sea water (Table VI, column 5). The value of the deviation for the nuclei that were placed in 60 per cent sea water only after being previously equilibrated with 80 per cent sea water is 12.75 per cent. The difference between the deviations is 1.64 per cent, which is equal to the difference between the averaged volumes of the nuclei in 100 per cent sea water (column 1).

In the case of the series in 70 and 50 per cent sea water, there is a difference between the deviations of 3.75 per cent (column 5) as a result of the two different methods of bringing the nuclei into 50 per cent sea water. The difference between the averaged volumes initially is only 0.84 per cent (column 1). Here the agreement is not as good as for the series in 80 and 60 per cent sea water, and it appears that the property of perfect semi-permeability may be lost at dilutions as great as 50 per cent sea water.

The degree to which the germinal vesicle obeys the Boyle-van't Hoff law may be determined from the data in Table V. Although biological variations of considerable magnitude occur (note, for example, the variations in the averages of the initial volumes, P_0V_0 , column 4), the following conclusions are perfectly clear. First, there is better agreement with the Boyle-van't Hoff law at the lesser than at the greater dilutions; compare the values of P_0V_0 with those for P_1V_1 (columns 4 and 5 respectively) for any one relative pressure (column 1). Secondly, the magnitude of the deviations is greater for the nucleus of the *Nereis* egg than for that of the *Arbacia* egg. Thus the entries in Table V show deviations ranging from 10 to 15 per cent in 70 per cent sea water, whereas comparable deviations for *Arbacia* nuclei occur only at dilutions as great as 50 per cent sea water. The value of the deviation for *Nereis* nuclei in 50 per cent sea water is of the order of 25 per cent (Table V, column 6; Table VI, column 5). Finally, it may be mentioned that the nuclei shrink in hypertonic solutions, although data have not been compiled to test quantitatively this aspect of the problem. It may be concluded, then, that the germinal vesicle of the *Nereis* egg behaves as an osmotic system, though not to the same high degree as that of the *Arbacia* egg.

It is appropriate at this point to discuss the meaning of " b ," the value of the osmotically inactive material, for these experiments.²

² Except where explicitly stated, the remainder of this article is concerned exclusively with the data for the *Arbacia* material. A complete set of statistical data for cells and nuclei is presented in Tables VII and VIII.

In a study of this sort it is desirable to have an exact idea of the variability of the material. The eggs chosen for experiment were restricted more or less to the

For one of the purposes of this work, in addition to the investigation of the osmotic properties of the nucleus, was to determine, if possible, the values of " b " for cells and nuclei at various stages during their growth. This would have given a physiological index of great importance for understanding intracellular growth. (A partial solution of this problem will be offered further on.) Unfortunately, the almost insurmountable difficulty of making great numbers of measurements on groups of cells of similar sizes was immediately encountered. Consequently it was necessary to abandon this particular phase of the investigation and to concentrate instead on determining the osmotic relationships of a rather heterogeneous population of cells and nuclei; the averaged volumes being used in the equations. Obviously, an "average" value of " b " for cells and nuclei of very different volumes has little or no physiological meaning.

Actually, the data for the nucleus indicate that there is an insignificant amount of osmotically inactive substance present. This is proved by testing statistically the agreement between the data and the Boyle-van't Hoff law. The analysis consists in determining the significance of the "difference between the means" (the values of P_0V_0 minus those for P_1V_1 for 80, 70, 60, and 50 per cent sea water). If the differences are not significant, then Boyle's law holds; and vice

later growth stages: the diameters of the cells vary from 65 to 80 μ ; those of their nuclei from 35 to 42 μ (column 4, Tables VII and VIII respectively). From the coefficient of variation, C , (column 6) it can be seen that initially the variability, representing entirely variations in sampling technique, is of the order of ± 10 per cent. This figure is the same for cells and nuclei, indicating that variations in the nuclear volumes are associated with variations in egg volumes—a deduction to be expected on *a priori* grounds. In addition, the data show that the variations in the equilibrium volumes in anisotonic solutions are determined to a considerable extent by the variations in the initial volumes. From this the unexpected result emerges that *the variation in the final volumes is independent of the concentration of the medium*.

Another set of statistics that is important is concerned with the correlation coefficient, $r_{V_0V_1}$. Indeed, the correlation between the initial and final volumes is in reality a measure of the degree of validity of the Boyle-van't Hoff law. For if the process followed this rule exactly, then theoretically a correlation coefficient, ρ , of $+1$ would be expected for the supply. The effect of the great variation found in the samples will act, however, to reduce the value of $r_{V_0V_1}$ considerably. In other words, the correlation will tend always to *underestimate* the degree of association.

Some idea of the variations encountered in the correlations can be determined by use of the regression equations and standard error of estimate (Table IX). These equations are extremely useful since they indicate the most likely value, expressed in cubic micra, that the volume of a cell or its nucleus will attain when immersed in a hypotonic solution. The coefficient of V_0 , the slope of the regression line, expresses, in cubic micra, the average change in V_1 accompanying unit change in V_0 . Evidently the greater the correlation between V_0 and V_1 in the different solutions, the more nearly alike the values of these coefficients will be. The data show that not only are the values of the slope very much alike for different media, but that they are quite similar for cells and nuclei interchangeably. The variations around the regression line are given by the standard error of estimate which may be interpreted in the same way as a standard deviation.

versa. Because of the high correlation involved (Table VIII, column 7), it is necessary to make this test as sensitive as possible. To this end the standard error of the difference has been calculated from the following formula (Treloar, 1939, pp. 146-148):

$$\text{S.E.}_{\bar{d}} = \sqrt{\text{S.E.}_{P_0V_0}^2 + \text{S.E.}_{P_1V_1}^2 - 2r_{V_0V_1} \cdot \text{S.E.}_{P_0V_0} \cdot \text{S.E.}_{P_1V_1}}$$

From the actual difference between the means and the standard error of the difference, the relative deviate has been determined and the probability read off from prepared tables.

When this test is applied to the data for the nucleus (Table VIII), it is found that the difference in the means of 0.02 for 80 per cent sea water can arise in 95 cases out of 100 merely through errors of random sampling. Such a difference, then, is insignificant and the simplified equation $P \cdot V = K$ suffices. On the other hand, this equation is no longer valid when the difference of 0.77 for 70 per cent sea water is considered, for here the 5 per cent level of significance is approached. In 60 and 50 per cent sea water the differences are highly significant. Nevertheless, the fact is inescapable that in 90 and 80 per cent sea water the value of " b " is zero,³ and so the nucleus cannot be said to contain any significant amount of osmotically inert material.

The conclusion that the nucleus contains an inappreciable amount of osmotically inactive substance requires qualification. Certain pertinent viscosity data in the literature may be interpreted as indicating that a considerable amount of dispersed material exists in the nucleus. For the viscosity of a solution is related in a complicated fashion to the volume fraction of the solute. The viscosity of the germinal vesicle of the *Echinus* egg, a form closely allied to *Arbacia*, has been variously determined as being from two to ten times that of water (Heilbrunn, 1928; Harris, 1939). Employing any of the numerous equations relating viscosity to the volume fraction of the solute

(the most satisfactory of which appears to be that of Kunitz, 1926: $\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4}$) estimates of φ (b ?) range from 10 to 40 per cent of the total volume. It should be noted that this treatment tacitly assumes that the term "osmotically inactive material," b , is synonymous with that of "volume fraction of solute," φ . This is, indeed, the conventional definition of " b " as employed by physiologists since its conception by Hamburger and Hardy. Actually, as measured by osmotic effects, the b -factor represents an *effective* rather than a real

³ The same conclusions apply in the case of the germinal vesicle of the *Nereis* egg. This is shown by the agreement between the values of P_0V_0 and P_1V_1 in 90 per cent sea water (Table V) and in 80 per cent sea water (Table VI, columns 1 and 2). In the latter case the value of the deviation is only 0.19 per cent.

volume, and as such might well be some multiple of φ . A real discrepancy exists between the values of " b " and of " φ " or some multiple thereof.

There are in the nucleus only two formed structures which might be expected to contribute to the osmotic "dead space." These are the chromosomes and the nucleolus. By analogy with the behavior of other chromosomes, those of the germinal vesicle should behave osmotically.⁴ The osmotic properties of the nucleolus will receive especial attention in a following section. In short, it hardly seems likely that any considerable proportion of " b " (or φ) can be due to the morphological constituents of the nucleus.

With respect to the cell as a whole, the situation is somewhat different. There can be no doubt that the differences between the means are real. Thus, for cells swollen in 80 per cent sea water, the chances are less than 1 : 10,000 that a difference of 7.92 could arise from sampling errors alone. One can calculate an "average" value of " b " and compensate for the deviations from the equation: $P_0V_0 = P_1V_1$. (Interestingly enough, the viscosity of the cytoplasm, as determined by centrifuge experiments, is probably very high—indicating a high value for φ .) The drift would still remain, although it would be smaller in magnitude. The largest deviation would again be found in the case of 50 per cent sea water. One could test for the significance of the difference between the means [values for $P_0(V_0 - b)$ minus those for $P_1(V_1 - b)$] as before. If the differences were insignificant, all the data would be accounted for by this simple modified equation and the analysis would be complete. If not, one would seek other causes to explain the discrepancies. It seems to me, however, that this sort of treatment is extremely deceptive in its implications, especially if no independent method for determining " b " exists.

In a previous section it was stated that a partial answer would be given to the question of the relation of the osmotic behavior of these immature ova and their germinal vesicles to their initial volumes. From the point of view of statistics the problem may be worded thus: Given the two variates, V_0 and V_1 , where V_1 is a function of V_0 , what relation does V_1 bear to V_0 as V_0 varies from low to high values?

Problems of this sort were investigated by Harris (1909). The solution is obtained from a consideration of the three possible cases for the values of r_{xz} as calculated by the formula

$$r_{xz} = \frac{r_{V_0V_1} - C_{V_0}/C_{V_1}}{\sqrt{(1 - r_{V_0V_1}^2) + (r_{V_0V_1} - C_{V_0}/C_{V_1})^2}}.$$

⁴ The giant chromosomes of the salivary gland cells of *Sciara coprophila* and of *Chironomus* sp. swell and shrink reversibly in anisotonic solutions; unpublished data.

The three cases that arise are: (1) when $r_{xz} > \text{zero}$, V_1 will tend to attain relatively larger values (i.e., obey the Boyle-van't Hoff law more closely) as V_0 increases from smaller to larger volumes; (2) when $r_{xz} < \text{zero}$, V_1 will tend to attain relatively smaller values (i.e., deviate more and more from the Boyle-van't Hoff law) as V_0 increases in value; and, (3) when $r_{xz} = \text{zero}$, it may be stated that small and large ova or small and large nuclei obey the Boyle-van't Hoff law to an equal degree.

The values of r_{xz} for all dilutions as calculated from the preceding formula are given in Tables VII and VIII, column 8. They are consistently negative in sign and of considerable magnitude, especially at the greater dilutions. The conclusion is drawn that smaller eggs and smaller nuclei (within the limited ranges of volumes investigated) tend to be better osmometers than larger eggs and larger nuclei.

TABLE VII
Immature eggs of *Arbacia punctulata*

Relative Pressures	Number of Cells	Averaged Volumes $\pm \text{P.E.}_M$ μ^3	Ranges μ^3	Standard Deviation, σ ; $\pm \text{P.E.}$ μ^3	Coefficient of Variation, C ; $\pm \text{P.E.}_C$	Correlation Coefficient, $r_{V_0V_1}$; $\pm \text{P.E.}_{r_{V_0V_1}}$	r_{xz}
0.8	V_0	198.76 ± 2.14	158.3–226.0	16.16 ± 1.51	8.13 ± 0.76	0.857 ± 0.035	–0.100
	V_1	238.55 ± 2.82	189.4–272.4	21.32 ± 1.99	8.94 ± 0.84		
0.7	V_0	217.73 ± 3.03	170.0–261.6	25.79 ± 2.14	11.84 ± 1.00	0.848 ± 0.033	–0.072
	V_1	283.65 ± 4.45	216.4–376.7	37.90 ± 3.15	13.36 ± 1.13		
0.6	V_0	208.81 ± 2.08	167.4–261.6	19.55 ± 1.47	9.36 ± 0.71	0.767 ± 0.040	–0.246
	V_1	307.98 ± 3.30	237.4–369.9	30.98 ± 2.34	10.06 ± 0.77		
0.5	V_0	213.98 ± 3.89	172.7–270.6	22.69 ± 1.86	10.60 ± 0.88	0.792 ± 0.043	–0.332
	V_1	356.34 ± 4.31	294.9–451.5	37.26 ± 3.04	10.46 ± 0.87		

During the course of this inquiry into the osmotic behavior of the germinal vesicle of the *Arbacia* egg, a number of observations were made on the osmotic properties of the nucleolus as well. The nucleolus is a small spherical body, about 12μ in diameter, appearing circular in optical section. It consists of a thick, highly refractive membrane enclosing a central lumen whose diameter is perhaps half that of the entire nucleolus. Less frequently the nucleolus has another form. It too is spherical, but smaller. The contents appear highly homogeneous, except for a few minute irrefractive “vesicles” enclosed within. It seems likely that this “solid” type of nucleolus is derived from the former by a rupture of the thick wall, followed by a loss of the contents of the large vacuole. The transformation is completed by a

TABLE VIII
Germinal vesicles of *Arbacia punctulata*

Relative Pressures	Number of Nuclei	Averaged Volumes $\pm \text{P.E.}_M$ μ^3	Ranges μ^3	Standard Deviation, σ ; $\pm \text{P.E.}_\sigma$ μ^3	Coefficient of Variation, C ; $\pm \text{P.E.}_C$	Correlation Coefficient, $r_{V_0 V_1}$; $\pm \text{P.E.}_{r_{V_0 V_1}}$	r_{xz}
0.8 V_0 V_1	46	27.48 $\pm$ 0.27 34.38 $\pm$ 0.36	22.3–33.4 27.4–44.9	2.71 $\pm$ 0.19 3.67 $\pm$ 0.26	9.86 $\pm$ 0.70 10.67 $\pm$ 0.76	0.838 $\pm$ 0.030	–0.155
0.7 V_0 V_1	51	27.52 $\pm$ 0.37 38.22 $\pm$ 0.53	19.0–36.1 26.3–51.8	3.95 $\pm$ 0.26 5.62 $\pm$ 0.38	14.35 $\pm$ 0.98 14.70 $\pm$ 1.00	0.808 $\pm$ 0.033	–0.275
0.6 V_0 V_1	53	28.02 $\pm$ 0.26 43.97 $\pm$ 0.44	23.3–36.6 33.8–55.5	2.81 $\pm$ 0.18 4.75 $\pm$ 0.31	10.03 $\pm$ 0.68 10.80 $\pm$ 0.72	0.667 $\pm$ 0.051	–0.332
0.5 V_0 V_1	48	28.75 $\pm$ 0.36 50.76 $\pm$ 0.60	20.6–37.6 39.6–62.1	3.75 $\pm$ 0.26 6.19 $\pm$ 0.43	13.04 $\pm$ 0.91 12.19 $\pm$ 0.85	0.751 $\pm$ 0.042	–0.435

refusion of the broken membrane, the entire process being accompanied by a general shrinking in volume. This phenomenon was actually observed in a swelling experiment with 80 per cent sea water. A few vesicles trapped within were the sole remnants of what had been the large central vacuole.

There is clear evidence that the nucleolus can swell and shrink reversibly. This is true whether one shrinks the cell in hypertonic solutions and then swells it back in 100 per cent sea water, or starts out by swelling it in diluted sea water and then shrinks it back in 100

TABLE IX
The estimation of equilibrium volumes, V_1 , from initial volumes V_0

Relative Pressures	Nuclei		Cells	
	Regression Equation μ^3	Standard Error of Estimate* μ^3	Regression Equation μ^3	Standard Error of Estimate* μ^3
0.8	$V_1 = 3.18 + 1.14 V_0$	± 1.48	$V_1 = 14.55 + 1.13 V_0$	± 8.35
0.7	$V_1 = 6.62 + 1.15 V_0$	± 2.32	$V_1 = 11.85 + 1.25 V_0$	± 13.67
0.6	$V_1 = 12.37 + 1.13 V_0$	± 2.10	$V_1 = 53.98 + 1.22 V_0$	± 12.56
0.5	$V_1 = 15.18 + 1.24 V_0$	± 2.48	$V_1 = 78.34 + 1.30 V_0$	± 13.87

* Standard error of estimate, $\pm \sigma_{V_0} \sqrt{1 - r_{V_0 V_1}^2}$.

per cent sea water. Just to give some idea of the magnitude of the changes that occur, three experiments with 60 per cent sea water will be presented; the figures should not be taken too seriously. Three cells whose nucleoli each had an initial volume of $0.8 \times 10^3 \mu^3$ in 100 per cent sea water were transferred to 60 per cent sea water. After a half-hour in this solution, two of the nucleoli had attained the volume of $1.3 \times 10^3 \mu^3$, and the other had increased in volume to $1.6 \times 10^3 \mu^3$. A half-hour after restoration to 100 per cent sea water two of the nucleoli had returned to their initial volume of $0.8 \times 10^3 \mu^3$ and the third had a volume of $0.9 \times 10^3 \mu^3$.

CONCLUSIONS AND SUMMARY

1. The nucleus (germinal vesicle) of the immature *Arbacia* egg closely approximates an osmometer in its behavior. The nuclear membrane gives clear evidence of being almost perfectly semi-permeable and only deviates from the Boyle-van't Hoff law at the greater dilutions. Data have also been presented on the osmotic properties of the nucleolus and the cell as a whole. There is some evidence that in the process of growth both cells and nuclei tend to become less perfect in their osmotic behavior.

2. The germinal vesicle of the mature, unfertilized egg of *Nereis limbata* also behaves as an osmometer, though not as perfectly as that of the immature *Arbacia* egg. It seems significant that these experiments fail to reveal any appreciable amount of osmotically inactive material in either of the nuclei. In this respect, however, it appears highly desirable that new and independent methods of experimental attack be devised for the study of the problem of the "non-solvent volume."

3. Some attention has been paid, in the case of the germinal vesicle of the *Nereis* egg, to the analysis of the phenomenon of leakage and an experimental method was devised for studying this factor. It was found that, for dilutions of sea water not greater than 60 per cent the volumes attained by the nuclei at equilibrium are independent of the path traversed. In 50 per cent sea water, however, there is some evidence that this rule does not apply. The implication of these results is discussed with reference to the occurrence of leakage in hypotonic solutions.

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THE INFLUENCE OF THE SINUS GLANDS ON GASTROLITH FORMATION IN THE CRAYFISH

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INTRODUCTION

The sinus glands located within the eyestalks of crustaceans have long been known to have an endocrine function in regulating pigmentary effectors. These glands, first described by Hanström (1933), have been the subject of various studies. But even before the sinus glands were known, there was evidence that the eyestalks formed a chromatophore-activating substance.

Decrease of the intermoult period after eyestalk removal seems to be hormonal in nature although the evidence in support of this is not yet complete. Megušar (1912) found that ecdysis occurred at more frequent intervals in eyestalkless than in normal *Astacus*. Brown and Cunningham (1939) made similar observations on the crayfish, *Cambarus immunis*, and Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941) on *Uca pugilator*. Smith (1940) showed that removal of both eyestalks in young crayfish shortened the intermoult period by slightly more than 30 per cent. Darby (1938) states that operative injury hastens the onset of moult in *Crangon armillatus*, but Smith (1940) showed that this was not true in young *Cambarus clarkii*. Plankemann (1935) reported that such diverse factors as starvation, increased metabolism, color of background and pH of sea-water might possibly affect the rate of moulting.

Since moulting and calcium metabolism are closely related phenomena, it might be expected that the mobilization and transport of calcium would also be regulated by a similar hormonal factor. Koller (1930) found that calcium in the moulted exoskeleton of *Crangon*, after eyestalk removal, was less than in the casts of normal animals and concluded that the so-called eyestalk hormone was definitely associated with calcium metabolism. Plankemann (1935) drew a similar conclusion from the known hormonal control of moulting in insects and the fact that the calcium content of the crustacean skeleton was modified during ecdysis.

Brown and Cunningham (1939) are the only ones so far to have tested the possibility of hormonal control of ecdysis in crustaceans by implantation of sinus glands after eyestalk removal. They found that the removal of the eyestalks increased the rate of moulting. When sinus glands were implanted into eyestalkless animals, there was a slight lengthening of the intermoult period. Low survival of their animals complicated their experiments so that while an endocrine factor in the regulation of ecdysis was indicated, conclusive evidence for the view was lacking.

In several of the *Astacura* gastroliths occur. They are rounded prominences, one on each side of the anterior region of the cardiac stomach, which are found in *Cambarus virilis* fully developed in the late spring.¹ The outer or lateral surface of each gastrolith is in contact with the epithelium of the stomach wall while the cuticular stomach lining covers the inner or medial surface. The outer surface of the gastrolith is smooth and convex, while the inner surface is flat.

Upon examination, a gastrolith can be seen to be composed of thin superimposed layers, the inner layers being parallel to the flat inner surface while the outer ones are concentric with the convex outer surface. A gastrolith is similar in structure to other parts of the exoskeleton except that the thicker layers are next to the epithelium.

During the moult the "stones" are freed by the casting off of the cuticular lining and are ground up and dissolved in the stomach, a new cuticle having been formed before shedding. According to Huxley (1889), in *Astacus fluviatilis* which are four years old, gastroliths begin to form forty days before ecdysis. The period is shorter in younger crayfish, being not more than ten days the first year after hatching. The process of their absorption takes twenty to thirty hours in young animals and seventy to eighty in the adult. Unless they are developed and then absorbed, ecdysis is not normally effected and the animal dies in the course of the process. Maluf (1940) states that gastroliths have about 3.16 per cent of the total ash present in the hard shell and are therefore of no significance after moulting. Numanoi (1939) describes the blood as more viscous and milky both during gastrolith formation and dissolution.

In an attempt to study the effect of the sinus glands on moulting in the crayfish, the gastroliths were most useful in determining oncoming moults, because their appearance precedes ecdysis and also since they are so easily detected by gross examination.

¹ Normal *Cambarus virilis*, collected in April and kept in the laboratory in running water, were observed to show a great burst of moulting in late May and early June. Of 75 of these animals, 38 moulted between May 27 and June 7.

The present investigation has been carried out with the hope of ascertaining more exactly the effect the sinus glands may have upon the process of ecdysis and especially of gastrolith formation in the crayfish. By the classical method of first removing the suspected gland and then reimplanting it, an attempt has been made to find what effect the gland has upon the formation of gastroliths which are indicative of oncoming moults.²

MATERIAL AND METHODS

In most instances observations were made on *Cambarus virilis*; however, *Cambarus clarkii* and *Cambarus immunis* were used in a few experiments. The specimens of *Cambarus virilis* were taken from local ponds and kept in running water in cement tanks except during experiments. The operated animals and controls were kept in some instances in individual finger bowls and in other instances in glass aquaria at room temperature (20–23° C.). The animals were not fed during experimental periods.

In removing the eyes, a thread was securely tied around the soft base of the eyestalk. With a small scalpel the part of the eyestalk just distal to the thread was severed. By this method bleeding was eliminated in most cases. At times some bleeding took place but a clot readily formed and the animals were not noticeably affected.

When a sinus gland was to be removed from an eyestalk in order to implant it into the same or another animal, the severed eyestalks were placed in Van Harreveld's (1936) crayfish perfusion fluid. The skeleton was split along the sides of the stalk by means of small bone forceps and the upper or dorsal part removed. This procedure exposed the sinus gland as a small bluish structure definitely differentiated from the mass of the eyestalk tissue. With the use of a dissecting microscope and fine needles it was relatively easy to disengage the gland and take it up in a pipette which was used in implanting the gland into the ventral abdominal region of the recipient animal.

In the histological study entire stomachs were removed and immersed in Bouin's fluid. They were rinsed in 70 per cent alcohol and then placed in 95 per cent and absolute alcohol. Cedarwood oil proved to be the best method of clearing before imbedding in paraffin. After sectioning they were stained with Mallory's triple stain and mounted.

Since the sinus glands, suspected of exerting hormonal control upon the moulting process in crayfish, are located in the eyestalks, the best

² This work was carried out under the direction of Dr. John H. Welsh, whose coöperation was most valuable.

approach seemed to be the removal of eyestalks from crayfish to determine whether their loss would cause the formation of gastroliths.

EYESTALK LIGATION EXPERIMENTS

In the first group of 29 *Cambarus virilis* both eyestalks were removed as mentioned above, the animals were killed after one or two weeks and their stomachs were examined. These experiments were started (using lots of 6 animals each) on September 28 and ran until December 7, 1940. In 25 of the 29 animals gastroliths were found, their size depending upon the weight of the animal and the length of time from operation to examination (Table I).

TABLE I

Animals with Two Eyestalks Removed

Eyes Off One Week

Total number examined	12
Gastroliths present in	10
Average weight of animals	35.5 grams
Average weight of gastroliths (pair)	27.1 mg.

Eyes Off Two Weeks

Total number examined	17
Gastroliths present in	15
Average weight of animals	19.9 grams
Average weight of gastroliths (pair)	10.1 mg.

Control Animals

Total number examined	20
Number of animals found having gastroliths	none

In the second group one eyestalk was removed from each of 23 *Cambarus virilis* in the manner described above. These animals were killed after one or two weeks and their stomachs examined. In no instance was there any indication of gastroliths or signs that they were forming, as was the case in all normal controls. These experiments were started September 28 and ran through November 8, 1940.

SINUS GLAND TRANSPLANTATION EXPERIMENTS

Since the removal of both eyestalks caused the animals to form gastroliths, it was decided to transplant sinus glands into eyestalkless crayfish to determine whether the glands might again exert their influence upon the animal's moulting process. If such were the case, these animals minus their eyestalks, and with implanted sinus glands should not form gastroliths.

In these experiments, all conducted between October 27, 1940 and March 7, 1941, the eyestalks were removed and two sinus glands implanted in the abdomen. In some instances the animals were examined one week after transplanting and in other instances additional transplants were made of two sinus glands at the beginning of the second week, and the animals were examined after two weeks. Twenty *Cambarus virilis* and 12 *Cambarus immunis* were used. Eleven of the animals died either on the day of the operation or the one following. Of those which survived, no traces of gastrolith formation were discernible upon examination of the stomachs.

TEMPERATURE EXPERIMENTS

Through the fall and winter, in *Cambarus virilis*, eyestalk removal resulted in the formation of gastroliths. The question arose as to whether or not a low temperature would prevent gastrolith formation. It had been noted earlier by Smith (1940) that temperature had a marked influence on moulting in young eyestalkless crayfish (*Cambarus clarkii*).

Animals with their eyestalks removed were placed in aquaria in cold rooms set at various temperatures. Others were placed at room temperature (about 21° C.). They were examined after two or three weeks to determine the influence of temperature upon the formation of gastroliths. Tables II and III indicate the results.

In the experiments conducted in December and January minute gastroliths were found in only 5 out of 23 animals kept at 3° to 15° C., while all animals kept at room temperature had gastroliths at the end of two weeks. In the April-May experiments (Table III), due to the approaching spring moult, many of the animals had gastroliths present before eyestalk removal. (Assumed after examination of a number of normal crayfish most of which possessed small gastroliths.) Little, if any, increase in size of the gastroliths occurred, however, in the eyestalkless animals kept for three weeks at 3°, 5° and 15° C., while the crayfish which survived for three weeks at room temperature showed fully formed gastroliths. Thus it is seen that temperatures of 15° C. or lower prevent or at least slow up the formation of gastroliths induced by eyestalk removal. This suggests the possibility that an enzyme system is involved in the removal of calcium from the exoskeleton and its deposition as gastroliths and that this enzyme process is inhibited during the winter, in part by the low temperature, and in part by an inhibiting hormone from the sinus glands.

TABLE II

Showing animals with two eyestalks removed subjected to various temperatures
(December-January)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
$^{\circ}\text{C.}$		<i>in weeks</i>	<i>in grams</i>	<i>in mg.</i>
3	1	3	29.1	0
"	2	"	23.8	0
"	3	"	21.3	0
"	4	"	20.7	0
"	5	"	27.2	0
8	6	"	28.1	0
"	7	"	24.2	2.2
"	8	"	27.0	0
"	9	"	27.2	0
"	10	"	38.0	2.8
"	11	"	26.0	0.6
11	12	2	14.1	0.2
"	13	"	17.5	0
"	14	"	17.1	0.2
"	15	"	13.8	0
"	16	"	14.0	0
"	17	"	23.3	0
15	18	"	24.9	0
"	19	"	21.3	0
"	20	"	20.7	0
"	21	"	23.1	0
"	22	"	17.6	0
"	23	"	16.8	0
20-23	24	"	24.1	9.6
"	25	"	29.3	0.9
"	26	"	27.2	20.6
"	27	"	19.5	3.0
"	28	"	21.3	21.4
"	29	"	25.9	20.0
"	30	"	18.2	52.9
"	31	"	26.4	57.0
"	32	"	21.5	7.4
"	33	"	28.3	20.2
"	34	"	34.3	0.4
"	35	"	23.5	7.8
"	36	"	29.2	9.3
"	37	"	34.5	9.2
"	38	"	3.9	37.6
"	39	"	22.2	21.3
"	40	"	4.0	21.2

HISTOLOGICAL STUDY

When it was seen that gastroliths were formed prior to moulting, whether this moulting were normal or induced by eyestalk removal, the question of the histological changes in the stomach epithelium prior to, and during gastrolith formation, arose. How soon after removal of

TABLE III

Showing animals with two eyestalks removed subjected to various temperatures (April-May)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
°C.		<i>in weeks</i>	<i>in grams</i>	<i>in mg.</i>
3	1	3	20.5	1.1
"	2	"	13.2	26.2
"	3	"	19.0	6.0
"	4	"	16.4	9.2
"	5	"	15.2	5.2
"	6	"	19.0	0
"	7	"	18.5	3.0
"	8	"	13.4	0.4
5	9	"	20.4	0
"	10	"	20.0	4.3
"	11	"	21.0	0.2
"	12	"	14.7	0
"	13	"	19.6	0
"	14	"	22.0	1.3
"	15	"	16.4	6.2
"	16	"	13.0	17.0
15	17	"	19.5	0.2
"	18	"	10.5	4.2
"	19	"	14.2	8.1
"	20	"	14.9	17.0
"	21	"	15.0	2.3
"	22	"	12.5	0
"	23	"	13.2	5.0
"	24	"	12.0	4.0
20-23	25	"	12.0	199.0
"	*26	"	11.0	141.0

* Four animals in this group died before the end of three weeks.

sinus glands is there a noticeable change in the stomach epithelium which secretes the gastroliths? What are these changes?

Regions of the stomach where gastroliths form were fixed and studied histologically. Figure 1 indicates the condition of the stomach lacking gastroliths. Figure 2 illustrates the epithelial changes observa-

ble in the same region when a gastrolith is starting to form. Twenty-four hours after eyestalk removal there is proliferation of the epithelial cells (Fig. 2) which were originally of a low columnar type (Fig. 1). Gradually these cells become tall columnar cells. There is a subsequent crowding of the sub-epithelial connective tissue. By the end of 48 hours the cells are tall, crowded together and show large prominent nuclei (Fig. 3). Sections made of stomachs of animals whose eyes had been removed for one or two weeks show much the same condition of the

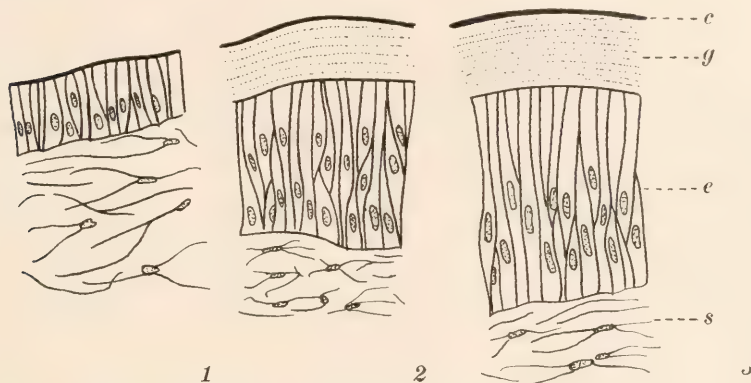


FIG. 1. Section of normal stomach of *Cambarus virilis* in region where gastrolith will form.

FIG. 2. Section showing gastrolith formation in stomach of *Cambarus virilis* 24 hours after eyestalk removal.

FIG. 3. Section showing gastrolith formation in stomach of *Cambarus virilis* 48 hours after eyestalk removal.

Abbreviations: C, cuticle; G, gastrolith; E, epithelium; S, subepithelial connective tissue.

epithelium as is illustrated by Fig. 3. Gastroliths begin to form 24 hours after eyestalk removal. However, Bouin's fluid, containing acetic acid dissolves much of the inorganic material of the gastroliths and makes a true histological picture difficult to reconstruct.

DISCUSSION

During the fall and winter normal animals show no gastroliths. Induced gastroliths, that is, those formed after eyestalk extirpation, were never found to be as large as those present in the spring and early summer during the normal moulting period. This was apparently due in part to the fact that gastroliths are from three to four weeks in their formation (forming much of their thickness during the last week) while

the experimental eyestalkless animals were ordinarily killed after a period of one or two weeks.

According to Robertson (1941) members of the suborders Anomura and Astacura do not appear to store calcium in the hepatopancreas. In Astacura gastroliths are formed in the foregut prior to moult. The gastroliths represent material which has been resorbed from the skeleton. A few hours after moulting they decrease in size and finally disappear, apparently being used as a source of calcium for the new skeleton.

Several investigators have studied the relation between calcium and moulting in Crustacea. The gastroliths, when present, and hepatopancreas, have been suspected as internal reserves drawn upon as the new skeleton is formed. Hecht (1914) found that once the skeleton of *Calinectes* was cast, the amount of calcium in the new individual was insufficient for the rebuilding of the new skeleton. Kleinholz, with the assistance of Emma Bourquin (1941), found that *Uca pugilator*, killed within five minutes after ecdysis, contained calcium equal to 1 per cent of the dry body-weight. The internal reserve constituted only 6 per cent of the total calcium content of the normal intermoult crab. The animal absorbed the balance of its calcium after moulting. Paul and Sharpe (1916) reported that in decapod Crustacea calcium was withdrawn from the hepatopancreas after ecdysis, and after the new shell was hardened, calcium was almost absent from this gland. Numanoi (1939) found the gastroliths of *Sesarma haematocheir* enlarged as moulting approached and disappeared after the moult. These changes he observed to take place concurrent with periodic changes in the blood calcium level.

Apparently the sinus gland acts as an agent controlling the passage of calcium, previous to moulting, from the exoskeleton into the blood where some of it is conveyed and deposited in the forming gastroliths. These act as reserve calcium which is utilized by the animal in order to harden partially its new skeleton until it can absorb more from its food and surroundings. Numanoi (1939) found that, in a species of *Sesarma*, when the calcium reserve is being dissolved after moulting, prior to being reprecipitated in the integument, blood calcium is raised to over forty times its normal level.

Why the sinus glands, which during the fall and winter inhibit moulting, do not do so in the spring is an interesting question. Apparently there is a seasonal variation in their activity which in the spring may be checked or inhibited by some other mechanism; also the low winter temperature would play some part in inhibiting gastrolith formation.

That the sinus glands are concerned in calcium metabolism of the crayfish during its moulting activity seems now to be reasonably certain.

The close relationship between the transfer of reserve calcium and moulting tends to point toward one mechanism as a control of both processes. The more intimate details of this mechanism still remain to be worked out.

SUMMARY

1. Removal of both eyestalks of *Cambarus virilis* and *Cambarus immunis* induces the formation of gastroliths at times of the year when they would not normally form. Removal of one eyestalk fails to induce gastrolith formation.

2. Transplantation of sinus glands into the abdominal region of eyestalkless crayfish prevents the formation of gastroliths.

3. Temperatures of 15° C. and lower prevent or greatly retard the formation of gastroliths in eyestalkless crayfish.

4. As early as twenty-four hours after eyestalk removal marked histological changes occur in the regions of the stomach which secrete the gastroliths. There is proliferation and increase in height of the epithelial cells and they have already begun the secretion of the gastroliths.

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THE CYTOLOGY OF THE ANTERIOR PITUITARY OF THE FOWL

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INTRODUCTORY STATEMENT

This paper is, for the most part, concerned with cytological questions, but it also attempts a correlation of cytological facts with experimental data. The study is by no means completed and will be carried on with the thought that an exhaustive study of one form is very much needed. The experimental side of the work has been done by Dr. W. R. Brene-man and due recognition is here given for that help. Without it, this study could not have been as extensive as it has been.

In a study of this kind, it is essential to know what happens in the normal course of events insofar as can be determined. These changes in themselves are interesting, but such knowledge becomes necessary as a basis for interpreting experimental results. One of the difficulties of the problem is in recognizing what the normal course of events is, for there is much variation due to variable and uncontrollable external and internal factors. This difficulty, however, can be overcome in part by the study of many pituitaries and this has been done (more than 800).

The White Leghorn chick has served as the basic material. Other varieties have been used to some extent for supplementary and comparative purposes, and where they are discussed in the text the fact will be made clear at that place. Severinghaus' modification of Nassonov has proved to be the best for cytological details. Nassonov and Helly were also used as well as Bouin. Various ways of staining were employed but the acid fuchsin methyl green method of Severinghaus gave the best results for most purposes. Other stains were used largely as checks. For a study of the Golgi material the usual osmic treatment was applied.

DEVELOPMENT AND MORPHOLOGY

On the bases of the distribution of the cells and the shape of the gland, the fully differentiated anterior pituitary has been described as divided into two parts. Rahn (1939), in particular, has accumulated considerable evidence in favor of this point of view and speaks of these

¹ Contribution No. 300 from the Zoölogical Laboratory.

two parts as the cephalic and caudal lobes. By some observers, including Rahn, this differentiation is carried back into the embryo as far as the sixth day. My own findings do not support this latter conclusion. The constrictions seen in the six-day embryonic gland are nothing more than irregular foldings of the wall of the oral evagination and may occur near the middle or anywhere else (Fig. 3, Plate I). At nine and fourteen days (Figs. 2 and 4, Plate I) there is even less evidence of a division. It is true that in the pituitary of fowls and of birds in general the acidophiles are usually distributed over only about half of the gland and when so distributed the two ends of the gland are very different in appearance. In some pituitaries, however, particularly from birds two to three months old, the acidophiles may extend over two-thirds or three-fourths (Fig. 5, Plate IV) of the entire gland and in such cases the lobe-like appearance is absent. This statement does not apply to a few small scattered cells, but to numerous large functional acidophiles.

CONTROLS

My studies of the control chick pituitaries (post hatching) have included the 3, 10, 15, 20, 25, 30, 35, 40 and 56-day periods. Some observations have been made at still later stages, but such studies are incomplete and must be supplemented with more material of known ages.

At three days the pituitary seems to be made up wholly of acidophiles and chromophobes. I say seems to be, for if any basophiles in early stages of transition are present, they cannot be sharply separated from the chromophobes and the non-granular acidophiles. Transitional stages, however, must be present, for functional basophiles are found at ten days. Rahn describes them in late embryonic development but I cannot confirm his observations.

Considerable work has been done in trying to discover one or more diagnostic characters by which cell types can be distinguished in early development. Size certainly does not answer the purpose in the fowl

PLATE I

FIGS. 1, 2, 3 and 4. Sagittal sections of embryonic pituitaries 3, 9, 8 and 14 days. In Fig. 2, the cavity of the hypophysial stalk has disappeared and in Fig. 4 the stalk itself has regressed in places. Note the place of attachment of the stalk in all figures. The posterior pituitary is omitted in Fig. 4.

FIG. 5. Sketch under low power, of a small area of a pituitary from an old fowl. The circles are outlines of the large golden spheres which appear in degenerating basophiles. The larger spheres are larger than the cells which means that the cells have broken down. Cell outlines are not shown. The drawing demonstrates particularly the large number of these spheres.

FIG. 6. A low-power sketch of a few of the cords with acini in a five-year-old Rhode Island Red cock. These acini appear shortly after two years of age in Barred Rock hens and become more numerous with age.



PLATE I

even though acidophiles and basophiles do in the end become larger than chromophobes. Neither does the shape of the cell, the shape and position of the Golgi material, the position of the nucleus, nor the position of the cell lend any help. In the large functional basophiles there are one or two (sometimes three) large spherical nucleoli which may appear vacuolated. The chromatin seldom is visible and if so, only faintly. This condition is in sharp contrast to that found in functional acidophiles where there are two or more small nucleoli, irregular in outline, and often somewhat obscured by irregular flocculent masses of chromatin. These two kinds of cells, then, as they approach the functional stage, can be distinguished fairly accurately on the basis of nuclear appearance. The nuclear picture in the chromophobes is very similar to that of the acidophiles. Unfortunately, transitional stages between the chromophobes and chromophiles cannot be clearly distinguished, probably due to the fact that changes are gradual. Staining reactions of nucleoli, nuclei, and cytoplasm help very little or none previous to the time of formation of secretory granules. In the ten-day embryo, Rahn distinguishes transitional cells on the basis of the degree of staining, the basophiles being much darker, but it seems to me such a method is very uncertain.

At three days the acidophiles are distributed throughout the pituitary but they are not alike in the two ends of the gland. In the caudal lobe, using Rahn's terminology, the cells are larger and possibly more columnar, although a conspicuous columnar arrangement does not enter until much later. As a rule, they stain more intensely, but this may be due to a greater accumulation of secretory granules. In fact, on the same slide I have seen cells in the cephalic end a brilliant carmine while those in the caudal end were orange. With the Altmann stain there is considerable variation in color, even in the same lobe when different slides are compared. Making allowance for all these variations, and for the fact that the caudal end cells may extend far into the cephalic region, the cells in the two ends still show differences. On the average, the cephalic end cells are smaller. They are more sparsely granulated and the granules may stain purple similar to the mitochondria. Differences in the two ends are particularly striking following fixation in Helly but Helly is not a good cytological fixative. Unfortunately Dawson and Friedgood's method (1937) by which they demonstrated two types of acidophiles in the rabbit and cat did not prove to be helpful, probably due to lack of skill on my part. Rahn definitely commits himself in favor of the point of view that there are two kinds of acidophiles, and on the basis of present evidence, I think this interpretation is correct. I shall refer to the caudal acidophiles as A1, the cephalic as A2.

Even at three days and probably at all later stages, there are cells with pycnotic nuclei. The degree of pycnosis is variable, grading from nuclei with only slight changes to others in which no structures are visible. Accompanying the pycnosis is a reduction in the size of the nucleus. The entire cell also becomes pycnotic, smaller in size, and modified as to shape (Figs. 6 and 7, Plate II). Are these pycnotic cells special cells which have developed directly from the chromophobes and if so, what could their function be? If they are not special cells, and I do not think they are, they must, in early post-hatching stages, be modified acidophiles for the basophiles remain undeveloped at three days. That these cells are modified acidophiles is clearly indicated by the fact that some of them have secretory granules in the cytoplasm. In later stages, after the basophiles become functional, they too may change in the same way (Figs. 2 to 5 and 8, Plate II).

Rahn describes pycnotic cells in the adult pituitary of the chick and identifies them as modified acidophiles. He says the greater and denser the amount of granulation, the darker and more pycnotic is the nucleus. My own observations show clearly that cells which exhibit the highest degree of pycnosis are completely or almost completely degranulated. They are cells which are regressing, probably toward chromophobes, possibly toward complete inactivity. In castrates where regressing acidophiles can be identified more accurately, pycnosis does not become so acute. My own point of view for the present is that cells with pycnotic nuclei as well as cells with pycnotic cytoplasm are acidophiles and basophiles which have passed the peak of activity. Speaking generally, active cells are not pycnotic, either with respect to the nucleus or the cytoplasm.

Ten-day Males

At ten days the acidophiles and chromophobes are still dominant. The general appearance and distribution of these cells have not changed greatly over the three-day period. The gland is larger, due in part to cell multiplication, and in part to cell enlargement. The major change at the ten-day period is the presence of large basophiles which are, without doubt, in the functional secretory stage. This is contrary to Rahn's statement that the basophiles are not functional until three weeks after hatching, but the evidence is clear and convincing. Besides, my observations are supported by the experimental evidence of Breneman (1937), who has shown by studies on gonad and comb growth that the testes have enlarged and that the male sex hormone is being secreted between ten and fifteen days. The basophiles, at this time, approach maximum size (see Fig. 1, *a* to *h*, plate IV, for comparative sizes). The

nucleus is large and spherical and the nucleolus (sometimes two) has become large in comparison to its former size. The cytoplasm and nucleus both stain well with methyl green. The cytoplasm, in different cells, varies somewhat in appearance. Sometimes it is uniform, sometimes slightly flocculent, and sometimes definitely granular. My conclusion that the basophiles are functional at ten days is based upon the fact that these cells are similar to functional basophiles in later stages of development as well as Breneman's experimental evidence. These functional basophiles occupy an area not definitely delimited and which can best be described as the central juxta-neural region, a region extending into both ends of the pituitary. More large basophiles are found in the caudal end, however, than in the cephalic. See Table I for body, comb, and gonad weights of 10-day control chicks.

Ten-day Female

As in the male, functional basophiles are present in the ten-day female. The general distribution of all cells in the two sexes is about the same, but the size of the gland in the female is slightly smaller. This difference may be due to slower division rate or again to fewer large basophiles. In some glands it appears as if the acidophiles were actually

PLATE II

FIG. 1. Outline sketch of a basophile from a 20-day control male.

FIGS. 2, 3, 4, 5, 6, 7, 8. Cells showing a series of retrogressive stages in both basophiles and acidophiles. Figs. 2, 3 and 8 are basophiles; Figs. 6 and 7 are acidophiles; Figs. 4 and 5 cannot be identified with certainty.

FIG. 9. An acidophile from a 30-day limited diet female showing a cluster of green granules adjacent to the nucleus.

FIG. 10. An acidophile from a 30-day limited diet male showing green granules but more scattered than in Fig. 9.

FIG. 11. A much modified basophile from an old (age unknown) Barred Rock cock. The cell is filled with spheres, which, following the acid fuchsin methyl green staining method, are golden yellow with a red periphery. The nucleus is dark and compressed.

FIG. 12. An acidophile in division.

FIG. 13. A basophile from a Buff Leghorn capon about seven months old. The numerous dark bodies in the cytoplasm are mitochondria.

FIG. 14. A basophile from an eight-week-old White Leghorn capon. Note the three large nucleoli.

FIG. 15. Basophile from an old White Rock hen. The dark spheres in the cytoplasm are mitochondria. The light spheres are golden yellow as in Fig. 11.

FIG. 16. A much modified basophile from a five-year-old Rhode Island Red cock. The large central sphere is golden yellow with a red periphery, similar to the smaller spheres in Figs. 15 and 16. The nucleus is pushed to the periphery of the cell.

FIG. 17. A basophile from a Barred Rock male about three months old. The golden bodies appear early in this bird and are irregular in outline instead of spherical as in Fig. 15.

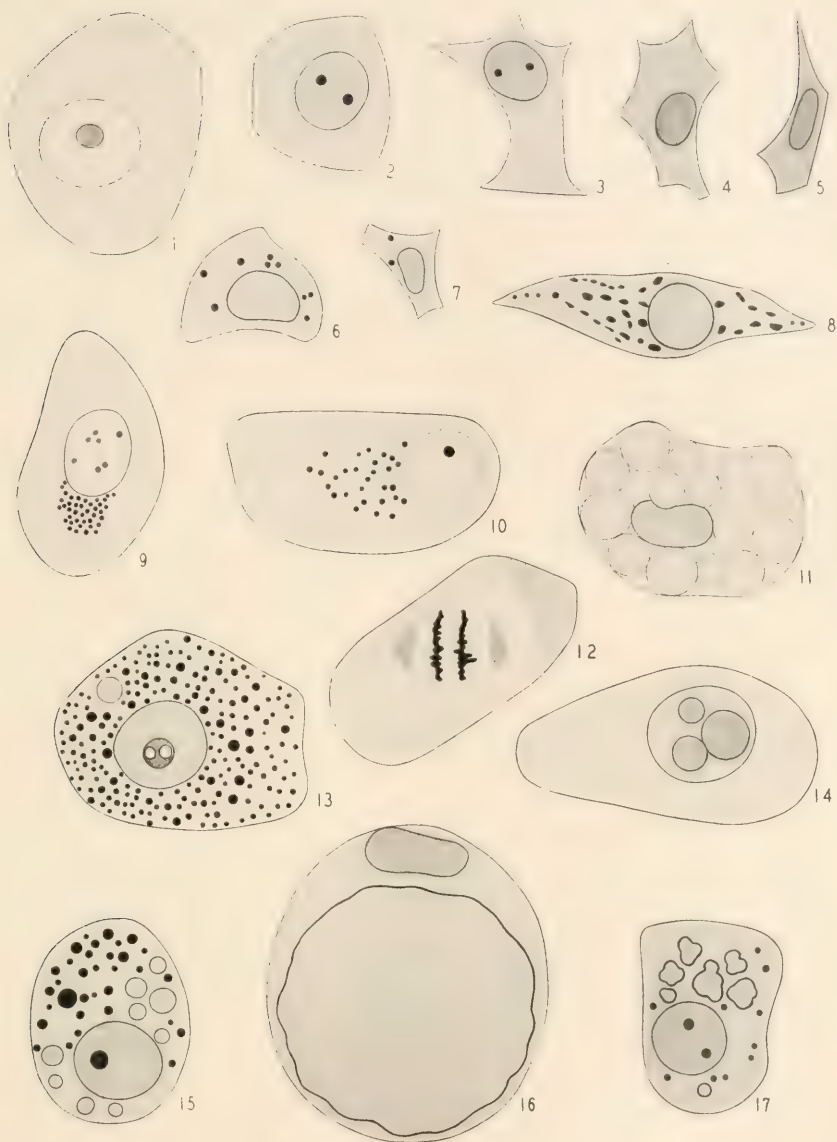


PLATE II

more numerous and even larger and more granular than in the male, but the evidence is not convincing.

Fifteen days and Older

Changes in the pituitary from 10 to 56 days are gradual. The gland increases in size due to the multiplication of all three types of cells and to the growth of the chromophiles. The rate of increase in the numbers of the cell types, however, is not the same, and hence the relative proportions change. While cell counts have not been attempted, the statements which follow are reasonably accurate, perhaps as accurate as they would have been had cells been counted. At 10 days, as stated, the chromophobes and acidophiles are the dominant types. Only a relatively

TABLE I

Body, comb, gonad weights and pituitary size in controls						
Age	Body Weight	Comb Weight	Gonad Weight	Comb Weight	Gonad Weight	Pituitary Size ³
<i>days</i>	<i>grams</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent of body weight</i>	<i>per cent of body weight</i>	
10	76.8	34.8	19.5	0.045	0.0254	2672.
15	106.0	115.4	36.9	0.109	0.0349	3378.
20	175.8	229.4	52.3	0.164	0.0291	4715.
25	190.7	316.6	61.0	0.166	0.0320	5302.
30	272.3	1336.9	86.0	0.491	0.0316	5958.
40	406.5	3740.0	179.7	0.922	0.0442	

³ See footnote 7, Table II.

few basophiles are present but after 10 days the basophiles increase in number more rapidly than the acidophiles. The relative proportions of basophiles and acidophiles, however, vary widely with variable external conditions such as food, living conditions, etc. Speaking generally, adverse conditions restrict basophilic development but have little effect upon the acidophiles. Sometimes the acidophiles actually stand out more prominently, but this impression may be due to the contrast with the small number of basophiles. Certainly the acidophiles are large and granular and the number is not decreased when chicks are reared under unfavorable conditions. One is tempted to suggest here that if the acidophiles secrete a growth-promoting hormone, they are secreting more actively to overcome the handicap of a poor environment.

After making allowances for variations, both external and internal, the basophiles increase in number as development progresses and under favorable conditions they become more numerous than the acidophiles at 56 days of age and probably earlier in some chicks. The exact relative

proportions, however, are not important. The fact which is important is that both kinds of cells are functioning and that the basophiles increase in number from the 10-day stage. During the laying periods of hens one and one-half years and older, the basophiles are the dominant cell type. As previously stated, functional basophiles appear first in the central juxta-neural area about ten days after hatching. From there they spread gradually into the posterior end and later (30 to 40 days old) into the anterior end. By 56 days they are found in all regions.

In Rhode Island Reds and Barred Plymouth Rocks no functional basophiles were present in the pituitaries of 20-day-old chicks. Many basophiles were present, however, in 25-day Barred Rocks. Twenty-five-day Rhode Island Reds were not studied. Here also we find a correlation between cytological observations and experimental data. Breneman (1941*b* and unpublished data) finds very little or no indication of sex hormones present in these two varieties at 20 days of age. These observations, in addition to the fact that Rhode Island Reds age earlier than White Leghorns, demonstrate clearly that varietal differences with respect to pituitary behavior exist.

Comparative pituitary sizes are of interest, particularly since they are in harmony with cell changes and with comb and gonad growth. See Table I.

GOLGI SUBSTANCE AND MITOCHONDRIA

As many biologists accept the theory that the Golgi substance is concerned with secretion and since Severinghaus has used the shape, size and position of this material as a means of identifying the acidophiles and basophiles, I wish to comment briefly on the behavior and significance of this substance as I have observed it in the fowl. My studies have been limited to osmic-treated pituitaries. Severinghaus has described two distinct types of Golgi substance or apparatus in the rat. In one type the substance "consists of a basket-like network closely applied to one side of the nucleus." The second type is somewhat spherical in shape, sometimes with one or two invaginations, and lies in the cytoplasm separated from the nucleus. The first type is characteristic of acidophiles, the second type of basophiles. The two types can be distinguished in the chromophobes.

In the fowl, unfortunately, the Golgi substance cannot be used as a distinguishing mark for the acidophiles and basophiles. An examination of this substance in the chromophobes such as those illustrated in Plate III shows clearly that there is much variation in form, shape, size and position. Even in granular basophiles and acidophiles, where there is no doubt of their identity, I cannot say that I can always identify these

cells by means of the Golgi substance. As the substance is difficult to describe with words, I prefer to let the figures, which have been carefully and accurately drawn to the same scale, speak for themselves. In Fig. 1, Plate III, is shown the Golgi substance in a large functional basophile and this appearance is general, except that in many of these cells, even after prolonged osmication, the substance is only faintly visible or cannot be seen. Figure 3, Plate III, shows an acidophile in which the Golgi substance is very similar in shape and position to that in Fig. 1. The osmication, however, is more pronounced. In other acidophiles, as illustrated in Figs. 4, 5, and 6, the substance is net-like. I have never seen this form in a functional basophile. In most or all of the columnar acidophiles the Golgi substance lies at the inner end, the end distal to the capillary. Apparently there is no orientation in the basophiles as illustrated in Fig. 3, Plate IV.

The presence of the Golgi substance in secretory and many other kinds of cells has raised questions no one of which has been answered with any degree of satisfaction. There are so few facts of which we are certain; of theories there are plenty. This is not the place to discuss these questions. I do wish to remark, however, that I find no convincing evidence that the Golgi substance is directly or even indirectly concerned with secretion. Secretory granules, so far as I can discover, may appear in regions of the cell remote from the Golgi substance.

PLATE III

With the exceptions of Figs. 2 and 17, all figures on the plate are from osmicated pituitaries and with the exception of Figs. 2, 7, and 17 were drawn to show size, shape, position and morphology of the Golgi substance in the cells of the anterior pituitary of the White Leghorn fowl. Most of the figures are from 13- and 18-day embryos and the cells drawn were selected at random, without regard to kind, in order to show as much variation as possible in the Golgi substance.

FIG. 1. A large basophile from a 47-day capon castrated at five days. There is very little accumulation of osmic. Hence the Golgi substance shows only faintly.

FIG. 2. A large basophile in a 46-day castrate which was injected daily from the thirty-fifth to the forty-fifth days with 0.5 mg. Oreton B. Note irregular shape of nucleolus.

FIGS. 3, 4, 5, and 6. Acidophiles. Figure 3 from a male 31 days old; Fig. 5 from a young male of unknown age, weight 2.5 lbs.; Figs. 4 and 6 from a female 45 days old. Note the net-like appearance of the Golgi substance in Figs. 4, 5 and 6.

FIG. 7. Acidophile from a 45-day female. Pituitary was treated with osmic. In the distal end of the cell (adjacent to blood vessel) is a large spherical gray mass with a smaller black sphere near the center. In Fig. 4 the same body is present but the gray sphere was not visible and the black sphere is in the form of a ring.

FIGS. 8, 9, 10, 12, 13, 15, 18, 19, 20, 21, 22, 23. Cells from an 18-day embryo. Drawn to show Golgi substance. Type of cells not identified. Most are evidently chromophobes; some are probably acidophiles.

FIGS. 11 and 14. Cells from a 13-day embryo. Golgi as in 18-day embryo.

FIG. 16. Chromophobe from a young male of unknown age. Weight 2.5 lbs.

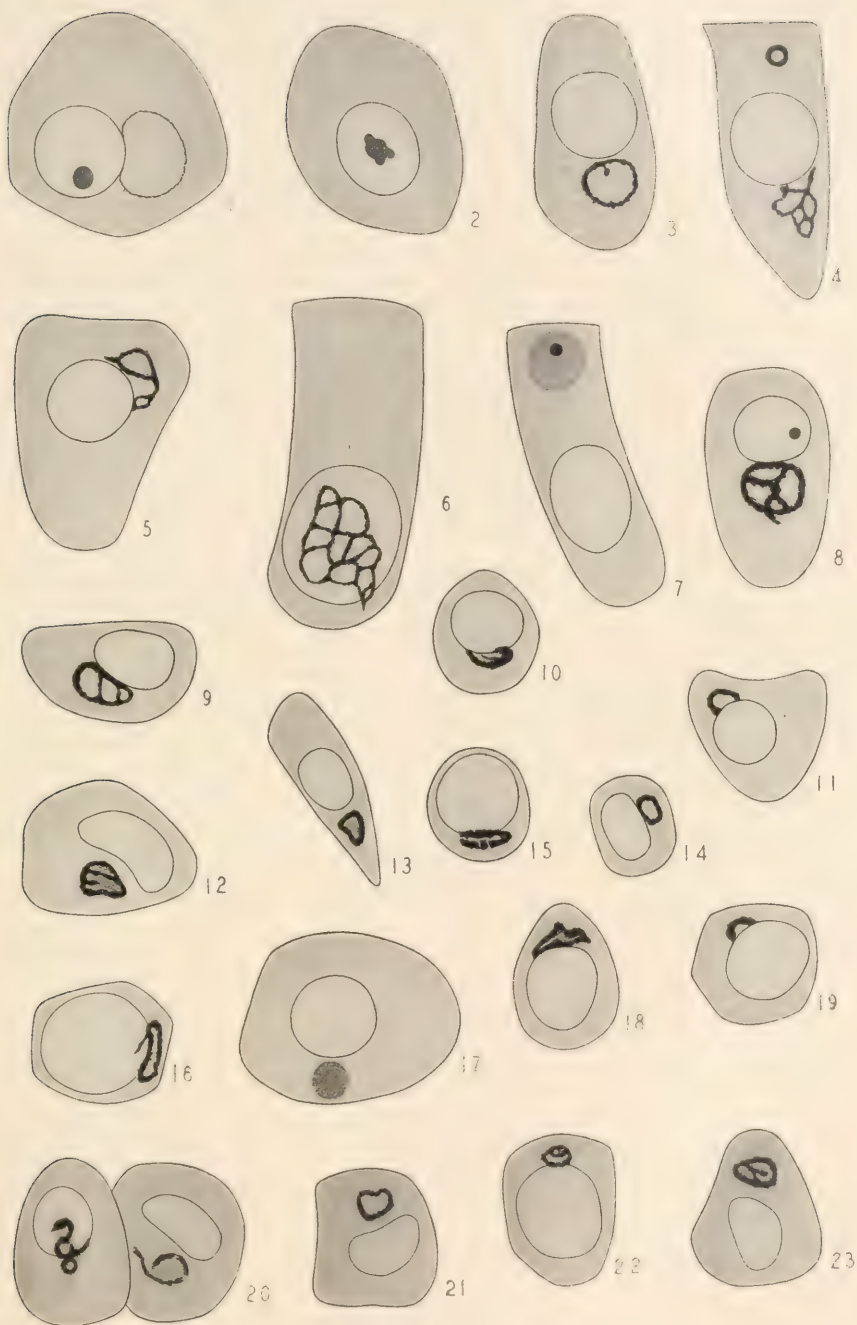


PLATE III

Figure 9, Plate II, illustrates an acidophile which was filled with secretory granules (not shown in the sketch). In addition to the usual secretory granules there is a cluster of larger granules lying near the nucleus. They stain green or bluish-green and are in sharp contrast to the red or yellowish secretory granules, when the acid fuchsin methyl green staining method is used. They may migrate into other parts of the cell as shown in Fig. 10. The position of the cluster, as in Fig. 9, indicates that the granules occupy or may occupy the same region as the Golgi substance. What are these granules? The first thought is that they are mitochondria for in other cells such as young insect ova (Payne, 1932) the mitochondria and the Golgi substance may in certain developmental stages be mingled together in a nuclear cap area (metabolic center of Schooley). Against this possibility, however, is the fact that mitochondria should, with the technique used, stain a purplish red. Further, mitochondria are usually distributed at random and can be definitely identified in the same cell with the green granules. Of course, mitochondria may change chemically and react differently at different times to the same stain. More definite statements about these green granules cannot be made at this time. I have, however, kept in mind the possibility that both acidophiles and basophiles may synthesize more than one kind of secretory granule.

Very little additional need be said about the mitochondria. They are more numerous in the basophiles than in the acidophiles, are distributed at random, and seem most numerous in the large basophiles of castrates (Fig. 13, Plate II). They are usually granular and variable in size, but may appear vesicular (Fig. 15, Plate II). In retrogressing cells, as in Figs. 2 to 8, Plate II, the mitochondria assume variable shapes and become less numerous with diminution in the size of the cells. No evidence as to their function has been obtained.

PLATE IV

FIG. 1. A series of drawings to show the relative sizes of the large basophiles at different ages in control males and females. Drawn to the same scale. (a) 10-day male; (b) 10-day female; (c) 15-day male; (d) 15-day female; (e) 20-day male; (f) 25-day male; (g) 30-day male; (h) 56-day male.

FIG. 2. A similar series of drawings to show basophilic size in castrates. Drawn to same scale as Fig. 1. (a) 20-day male; (b) 30-day male; (c) 35-day male; (d) 56-day male; (e) 90-day female.

FIG. 3. Basophiles from 47-day castrate male. Drawn to show shape of cells and position of nucleus and Golgi substance within the cells. Note irregularity with respect to these points.

FIG. 4. Saggital section of a 5-day embryonic pituitary. Note folds and secondary oral evaginations.

FIG. 5. Saggital section through middle of pituitary of male chick 10 weeks old. The dotted line gives the boundary between two types of acidophiles.

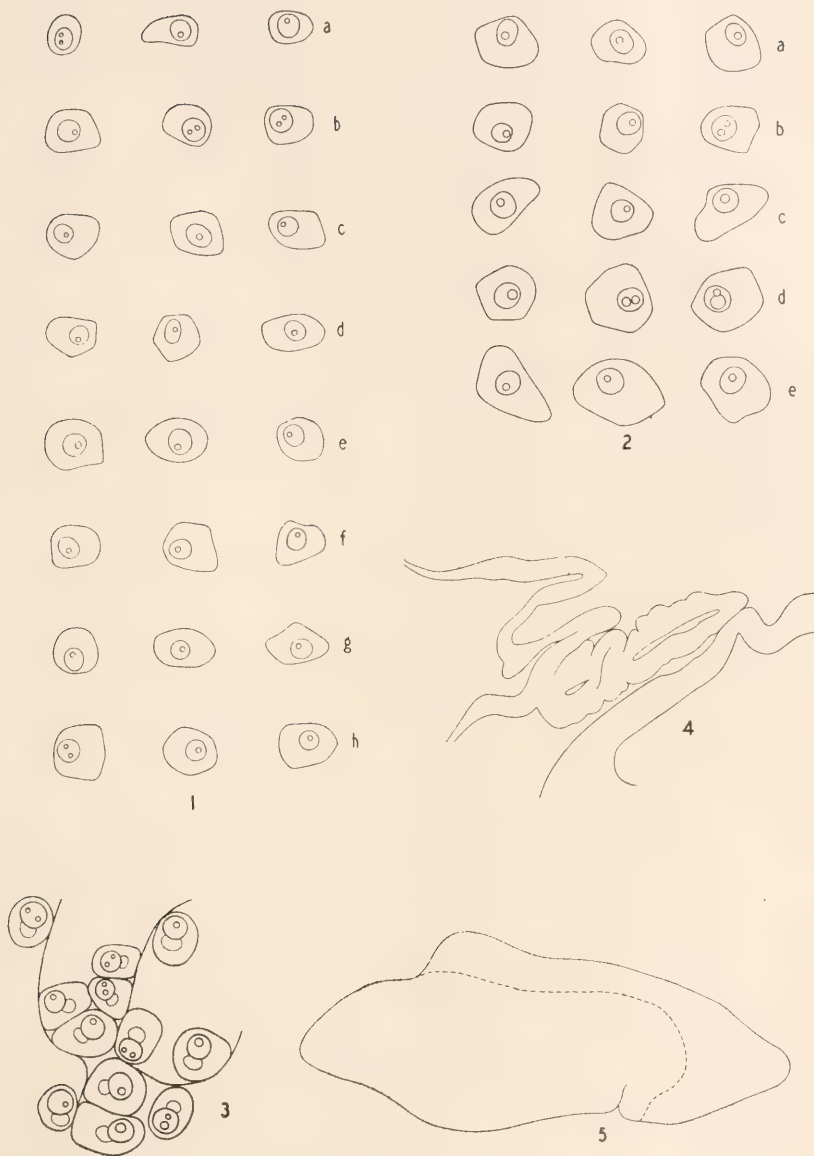


PLATE IV

AN UNKNOWN STRUCTURE

In one female 45 days old, the pituitary of which had been osmicated, an unusual structure was found in many of the acidophiles. Other than in this one pituitary it has not been seen. The structure (Figs. 4 and 7, Plate III) which is sometimes double appears as a black ring or sphere lying within a dark gray more or less spherical area, the whole of which lies in the peripheral end (toward the blood vessel) of the columnar-shaped acidophile. Since the Golgi substance lies in the opposite end of the cell and since there is no connection between the two, it seems independent of the Golgi substance. Neither does there seem to be any possible mitochondrial connection. In some cells the gray area does not show, due probably to uneven osmication or to uneven extraction of the osmic. Such irregularities in osmicated material are common.

A single instance of this kind might lead one to think that the structure is an artifact, but this does not seem possible. More likely it is a structure which becomes visible only when the cytological treatment, including osmication, is favorable. As to what possible function it might have, I make no suggestions.

LIMITED DIET BIRDS

Breneman (1941*a* and 1941*b*) has performed several experiments with limited diet birds. We mean by "limited diet" a limitation in quantity of food. After hatching, the chicks were fed on the second and on alternate days. In some experiments with older birds, food restriction was even more drastic. As the intake varied in different individuals, the responses to the limited diet treatment were variable. Pituitaries from 15-, 20-, 30-, and 31-day limited diet chicks have been studied, and also pituitaries from 15- and 30-day limited diet castrates. In all the limited diet non-castrate chicks the general effects are the same. The basophiles are undeveloped or restricted in comparison with the normal diet controls, and the acidophiles are as numerous and as well developed as in the controls. They actually seem, in some instances, to be more numerous than in controls but the different appearance is probably due to the changed proportions of the two types of cells. The pituitaries of the limited diet chicks are, without exception, smaller than in the normal diet controls. For relative sizes see Table II.

According to Breneman's data (1941*b*), the body, comb, and gonad weights of limited diet male chicks are much less than in controls (Table III). Since the basophiles of the pituitary are greatly restricted in their development in limited diet chicks, we have a direct correlation between the low gonad weight and the meagre development of the

basophiles. The natural inference is that in limited diet chicks the gonads remain small, in part, at least, because of the limited amount of gonadotropic hormone present.

In contrast to the low weight of comb and gonads in limited diet chicks, Breneman (1941b) found the intestine longer and heavier in

TABLE II

Size⁷ relations of pituitaries (from males)

Age	Controls	+5 I.U. Estrone	+100 I.U. Estrone	+500 I.U. Estrone	+0.0005 mg. Stil- bestrol	+0.2 mg. Stil- bestrol	+0.125 mg. O.B	+0.01 mg. O.B	+2 units Prolactin
20 day emb.	1860								
3 day	2068								
10	2672.5								
15	3378.5	2740		2415			4216.6		3552.5
20	4715		3223	3696.6					
25	5302								
30	5958.3	4362.5		3926.6	4020	3525		4970 ⁴	
35	6512.5								
56	9400								

Age	Cas- trates	Castrate +100 I.U. Estrone	Castrate +5 I.U. Estrone	Castrate +0.125 mg. O.B	Castrate +0.05 mg. O.B ³
20 day	5376.3	4843.3		4120	
25	6105				
30	7084		6205		6460
35	7540				
56	13832.5				

Age	Limited diet	L.D. ⁶ Cas- trate	L.D. +500 I.U. Estrone	L.D. ♂ +0.01 mg. O.B	L.D. ♀ +0.01 mg. O.B	L.D. Castrate +0.01 mg. O.B	L.D. +5 I.U. Pro- lactin	L.D. +0.07 mg. O.B	L.D. +pellets T.P.
15 day	2095.6	2055		2760	2358	2527		2843.3	2053.3
20	3110		2595				3600		
30	4288	5610							

⁴ Thirty-one days old.

⁵ Oreton B (dihydroandrosterone benzoate).

⁶ Limited diet.

⁷ For comparing gland size, outlines of median saggital sections were drawn with a camera lucida at an enlargement of 58 diameters. These drawings were then traced with a planimeter to get the areas in sq. mm. The figure given in the table is the mean size for several glands.

proportion to body weight than in controls. From this observation it seems clear that the intestinal growth is not dependent upon the gonadotropic hormone as is the gonad and indirectly the comb. Riddle and his co-workers have shown that prolactin induces an overgrowth of liver and intestine. Is it possible that prolactin is the cause of splanchnomegaly in the White Leghorn limited diet birds? If prolactin is secreted by the acidophiles, as suggested by Riddle and Schooley, we have in the White Leghorn limited diet chicks the cellular basis for an explanation of splanchnomegaly. While the basophiles are suppressed or retarded, the acidophiles are as prominent or more so than in controls.

If this interpretation is correct, then we should also find splanchnomegaly in estrone-injected chicks where the acidophiles are well developed and the basophiles retarded. Breneman (unpublished data) has compared estrone-injected chicks with controls. He finds that in the experimental birds intestinal weight and length and liver weight are higher in proportion to body weight by 30 to 90 per cent.

In castrates we find the reverse condition; that is, the basophiles are accelerated in their development while the acidophiles are retarded. There should be no splanchnomegaly in castrates and Breneman's preliminary data indicate that intestinal size and liver weight are less in proportion to body weight than in controls.

Limited diet restricts the development of the pituitary, affecting particularly the basophiles. Castration speeds up the development of the basophiles and causes degranulation and regression of the acidophiles. Pituitaries of thirty-day castrates fed on a limited diet were intermediate in size (5610) between pituitaries from castrates (7967.5) and those from limited diet chicks (4288) of the same age (Table III). The two types of chromophilic cells are also intermediate in their development, the basophiles being more highly developed than in limited diet birds and less well developed than in castrates. The acidophiles are better developed than in castrates and less so than in limited diet birds. The 15-day limited diet castrates were very similar to 15-day limited diet chicks.

CASTRATE PITUITARIES

That the removal of the sex glands has marked effects upon the pituitary is well known. While there are conflicting statements in the literature, Severinghaus (1937) says in his review, "It seems there is an increasing unanimity in the belief that the major change in the castrate pituitary is an increase in the basophiles." He further says that most authors have reported a reduction in size and degranulation of the acidophiles. Schooley (1937), working with castrate pigeons, described

an increase in the size of the pituitary. At 1.3 months after castration, normal and castrate pituitaries weighed the same. At 1.7 months castrate pituitaries were 50 per cent heavier than pituitaries from controls. The basophiles had increased in number and size. By 2.5 months the acidophiles were few and atrophic.

To date my studies of pituitaries of castrate chicks have been limited for the most part to birds from 10 to 56 days old. A few 90-day capons reared under adverse conditions were examined and a few Buff Leghorns weighing about four pounds each and probably about seven or eight months old were checked especially for signet-ring cells and aging effects. Most castrations were performed between 36 hours and 6 days after hatching—a few at 14 days.

At 10 days, even though castrated at 36 hours, there were few, if any, significant changes in the pituitary. It is possible, of course, that a study of additional material might show some effects at 10 days, for at 12 days, basophilic cells were definitely increasing in size and at 15 days the changes were very evident. At 20 days, many more basophilic cells were affected and the nucleoli of such cells had become enlarged. The basophiles in the mid-juxta neural region were first affected. The effect extended next into the posterior end and finally into the anterior. In all later stages studied including 25, 30, 35 and 56-day-old castrates, the basophiles continued to increase not only in number but in size as well (see Fig. 2, Plate IV for comparative sizes of these cells at different ages). The nucleoli also continued to enlarge during this period. In extreme cases it (sometimes two or three) occupied most of the nuclear space. In fact the enlarged nucleoli give the best landmark of the castrate pituitary (Fig. 14, Plate II). The Golgi material appeared much the same as in actively secreting basophiles of controls.

The mitochondria, on the other hand, were larger and more numerous, especially in the large basophiles of older castrates (Fig. 13, Plate II). Not only do the mitochondria increase in number and size, but they may become vesicular and fuse as in old fowls. This observation leads one to question whether aging effects may not appear earlier in castrate pituitaries than in non-castrate pituitaries and to suggest that such changes might account for the results of Bates, Lahr and Riddle (1935), who report that the pituitary of the steer showed a low gonadotropic potency when compared to pituitaries from embryos, calves, adult bulls, normal cows and cows in early and late pregnancy.

A second major change in castrate pituitaries, perhaps equally marked and equally significant as that which occurs in the basophiles, is the regression of the acidophiles. In controls these cells are very numerous in young chicks at the time of castration. Following castration de-

TABLE III

Average weights¹⁰ of body, comb and gonads of controls and experimental White Leghorn male chicks

Treatment		Age				
		10-day	15-day	20-day	25-day	30-day
Controls	No. used	32	18	25	29	30
	body	76.8	106	172.8	190.7	272.3
	comb	34.8	115.4	229.48	316.6	1336.9
	gonad	19.5	36.9	52.36	61.02	86.05
+5 I.U. estrone	No. used		4			16
	body		107.5			245.4
	comb		43.6			947.1
	gonad days injected		33.7 4-14			75.5 2-39
+100 I.U. estrone	No. used			14		10
	body			125.5		214.2
	comb			50.3		620.
	gonad days injected			20.7 3-19		51.25 3-29
+500 I.U. estrone	No. used		4	14		9
	body		99.5	119.9		221.7
	comb		26.5	39.6		251.7
	gonad days injected		16.2 4-14	14.7 3-19		49.2 3-29
+0.01 mg. O.B. ⁹	No. used					10
	body					239.1
	comb					1412.5
	gonad days injected					72.1 3-29
+0.0005 mg. Stilbestrol	No. used					12
	body					236.3
	comb					779.6
	gonad days injected					54.5 3-29
+0.2 mg. Stilbestrol	No. used					12
	body					235.6
	comb					123.7
	gonad days injected					31.2 3-29
Limited diet +0.01 mg. T.P. ⁸ Third day +1.5 mg. testosterone in pellet form	No. used		12			
	body		50.7			
	comb		324.2			
	gonad days injected		6.8 4-14			

TABLE III—*Continued*

Treatment		Age				
		10-day	15-day	20-day	25-day	30-day
Limited diet +0.07 mg. O.B	No. used body comb gonad days injected		7 63.4 198.5 15.3 3-14			
Castrates	No. used body comb				12 230.1 123.1	9 286.7 266.4
Castrates +5 I.U. estrone	No. used body comb days injected					7 243. 217.4
Castrates +0.05 mg. O.B	No. used body comb days injected					10 251.6 1374.8 10-29
Limited diet	No. used body comb gonad		31 61.2 23.9 15.9	12 74.1 24.9 15.3	30 95.5 42.1 17.7	27 158.9 235.1 51.0
Limited diet cas- trates	No. used body comb		7 57.1 17.6			5 124.2 62.3
Limited diet cas- trates +0.01 mg. O.B	No. used body comb days injected		7 56.2 23.7 5-14			
Limited diet +500 I.U. es- trone	No. used body comb gonad days injected			14 76.7 20.0 9.0 3-19		
+2 I.U. Prolactin	No. used body comb gonad days injected			14 132.8 120.0 34.9 3-19		

⁸ Testosterone-propionate.⁹ Oreton B (dihydroandrosterone-benzoate).¹⁰ Body weight in grams; comb and gonad weights in milligrams.

granulation takes place and then the cells gradually regress. In some castrate pituitaries only a few granular acidophiles persist, and as many of the chromophobes have changed into basophiles, the gland is composed largely of basophiles.

At all stages studied castrate pituitaries were larger than the pituitaries of controls (Table II) and as the acidophiles had regressed and the chromophobes had not multiplied, the enlargement was due to the increase in number and growth of the basophiles.

No signet-ring cells have been observed in any castrate chicks, even in the Buff Leghorns seven or eight months old.

While only a few pituitaries from gonadectomized females have been studied, it is clear that changes are similar in both sexes.

There is considerable variability with respect to the changes in castrate pituitaries. This is true even though the chicks are fed the same food, kept in the same room and the same cages. Those carried through at the same time, however, are more nearly alike. Adverse conditions retard the changes in both basophiles and acidophiles. One lot of castrates was kept in the country for 90 days. They were not well cared for and disease killed a number of the birds. Pituitaries from these capons showed fewer modified basophiles and the acidophiles had regressed much less than in younger castrates reared under better conditions.

Another lot of castrates was fed on a limited diet (limited in quantity) for 30 days. The size of the pituitaries from these chicks was even less than in 30-day controls and castration changes in both basophiles and acidophiles were retarded. For relative sizes of pituitaries, see Table II.

INJECTIONS OF ANDROGENS AND ESTROGENS

Because of the many conflicting observations and interpretations of the pituitary gonad relationships, Breneman, in a series of papers, partly published and partly unpublished, has sought to get further data on the problems by injecting varying amounts of both androgens and estrogens into normal or untreated chicks, castrates, and limited diet chicks. I shall describe the cytological changes in the pituitaries following injections and attempt a correlation of these observations with the experimental facts.

As previously described, functional basophiles are present in chicks 10 days after hatching. Acidophiles are well developed and filled with granules at the time of hatching. The size and number of these acidophiles increase as the gland enlarges. The basophiles also gradually increase in number as growth progresses, but since there were but few

such cells at 10 days, the number of basophiles increases relatively at a more rapid rate than the acidophiles.

Effects of Androgens on Normal Chicks

Two experiments were performed to test the effects of Oretone B (dihydroandrosterone-benzoate) on the pituitaries of normal birds. First, chicks were injected daily with 0.01 mg. Oretone B from the third to the twenty-ninth days after hatching and killed on the thirty-first day. Second, chicks were injected daily with 0.125 mg. Oretone B from the fifth to the ninth and from the eleventh to the fourteenth days after hatching and killed on the twentieth day.

In the 31-day-old birds of the first experiment, many basophiles had developed to the point where they were functional, but the number was not as great as in uninjected controls. The acidophiles still stood out prominently. Most were filled with granules while some had degranulated. The pituitaries were smaller than in 30-day uninjected controls, the ratio being 4970 to 5958.

In the second experiment, the amount of Oretone B was greatly increased (0.125 mg.) but the injection period was restricted. This greater dosage did not produce effects which were very different from those of the lesser dosage. There were some functional basophiles in these 20-day chicks, but the number was relatively smaller than in uninjected controls.

As in the first experiment, the pituitaries were smaller than in controls of the same age, 4216 to 4715.

Breneman (1937) finds that the testes are smaller in androgen-injected chicks than in controls and this is the result expected since the basophiles are reduced following androgen injections.

Castrates Injected with Oretone B

The following data are far from adequate but they are indicative. Twenty-day-old chicks were castrated on the fifth day and injected daily with .125 mg. Oretone B (dihydroandrosterone-benzoate) on the fifth to ninth and the eleventh to fourteenth days. There were no indications of castration effects on the pituitaries. In fact, the glands were smaller than those of 20-day controls. According to the method adopted for measuring size, these figures are 4715 for controls and 4120 for the injected castrates.

Thirty-day-old chicks castrated on the fifth day were injected daily from the tenth to the twenty-ninth days with 0.05 mg. Oretone B. The pituitaries showed a marked increase over those of controls in the number

and size of the basophilic cells, and they also showed a degranulation and recession of the acidophiles. The pituitaries were decidedly castrate-like. This is true with respect to the size of the gland as well as the cellular picture. The relative sizes of the pituitaries were, castrates 7084, controls 5958, injected castrates 6460. The relative weights of the combs are also interesting. For the 30-day controls the average weight was 1336.9 mg., for the 30-day castrates, 266.4 mg., and for the injected birds, 1374.8 mg. Of course the comb growth of the injected birds is due to the direct effects of the injected Oreton B. This increase demonstrates, however, that the dosage is large enough to be 100 per cent effective with respect to comb growth, and hence might have been expected to have had more effect on the pituitary than was actually the case.

These two experiments, while not conclusive, demonstrate that castration effects can be modified and possibly prevented by injections of Oreton B.

Effects of Estrogens on Normal Chicks

Estrogens were injected in varying dosages and for different periods of time into normal birds. Fifteen, 20-, and 30-day age groups were used. For the 15-day group, injections were made daily from the fourth to fourteenth days; for the 20-day group from the third to the nineteenth days; and for the 30-day group from the third to the twenty-ninth days. Three dosage levels were used, 5, 100, and 500 I. U. The major effects for all dosages and for all ages were the retardation of the development of functional basophiles and the restriction of the size of the pituitaries. The extent of the effects varied with the dosage, and with one exception results are in proportion to the quantity of estrone injected, the greater the quantity, the more pronounced the effects. Too much stress, however, should not be laid upon this exception for the size given is an average for only three pituitaries. Considering the variations to be found, it is remarkable that results are as consistent as they are. At 15 days pituitary size for controls was 3378.5; for chicks injected with 5 I. U. estrone, 2740; and for chicks injected with 500 I. U. estrone, 2415. At 20 days pituitary size for controls was 4715; for chicks injected with 100 I. U. estrone, 3223; and for chicks injected with 500 I. U. estrone, 3696.6. At 30 days pituitary size for controls was 5958.3; for chicks injected with 5 I. U. estrone, 4362.5; and for chicks injected with 500 I. U. estrone, 3926.6 (Table II).

As previously stated, functional basophiles are present in 10-day controls and the number increases with age. In 15-day chicks injected with 5 I. U. estrone, functional basophiles are present but when compared with controls the number is reduced; when injected with 500 I. U. estrone

no functional basophiles are present. These observations are not only consistent with respect to comparative pituitary sizes but also with respect to comb and gonad weights (Table III). In 10-day controls the average comb weight was 34.8 mg. and the gonad weight 19.5 mg. At 15 days these weights had increased to 115.4 and 36.9 respectively. The weights of comb and gonad in 15-day chicks injected with 5 I. U. estrone were 43.6 and 33.7. The gonad weights in these two 15-day-old groups are similar, 36.9 and 33.7. Comb weights, on the other hand, are very different, 115.4 for controls as against 43.6 for the injected birds. As comb growth is due largely to the effects of androgens and as the secretion of male hormone by the testes is dependent upon the action of gonadotropic hormone secreted by the basophiles of the pituitary, comb growth is indirectly a measure of basophilic activity as well as a measure of male hormone output. On the basis of comb growth, then, the basophiles should have been inactive in the 15-day estrone-injected birds, and by direct observation they were inactive.

At 20 days the comb in controls has increased in size to 229.48 mg. and the gonad to 52.36 mg. These weights in 20-day birds injected with 100 I. U. estrone were 50.3 mg. and 20.7 mg. When injected with 500 I. U. estrone they were 39.6 mg. and 14.7 mg. In both these experimental groups the basophiles were undeveloped and the small sizes of the combs and gonads indicate clearly that little or no gonadotropic hormone could have been released. It should be noted that the 500-unit dosage produces a greater inhibitive action than the 100-unit dosage, a fact which makes me think that the greater size recorded for the pituitaries of the 500-unit dosage is due to variation in gland size. If greater numbers of pituitaries had been measured, this inconsistency probably would not have occurred.

The combs in 30-day controls averaged 1336.9 mg. and the testes 86.05 mg. These weights are marked increases over 20-day controls and they demonstrate the high potency of endogenous gonadotropic and male hormones at this time. In 30-day chicks injected with 5 I. U. estrone, comb and gonad weights were 947.1 and 75.5; when injected with 100 I. U. estrone, these weights were 620.0² and 51.25,² and when injected with 500 I. U., they were 251.7 and 49.2. In all three experiments there is a reduction in size of both comb and testis. The testicular weights, however, with the 100 and 500 unit dosages, were essentially the same. The pituitary sizes for the controls, 5- and 500-unit injection groups, were 5958.3, 4362.5 and 3926.6 respectively. The basophilic picture is in accord with these facts. There are fewer large basophiles in the 5-unit

² These weights were for 31 days instead of 30 and hence the figures are slightly higher than they should be.

estrone-injected chicks than in controls, and there are still fewer large basophiles in the 100- and 500-unit injected groups.

Breneman has performed two experiments with daily injections of Stilbestrol from the third to the twenty-ninth days. Two dosage levels were used, 0.0005 mg. and 0.2 mg. The effects were similar to those obtained when 100 and 500 I. U. of estrone were injected. See Table II for comparative pituitary sizes and Table III for weights of combs and testes.

Limited Diet Chicks Injected with Estrone

Two lots of limited diet chicks were injected with estrone daily from the third to the nineteenth days and killed on the twentieth day. One lot was given daily injections of 100 I. U. and the second 500 I. U. Since estrone retards the development of the basophiles and since a limited diet does likewise, we have two forces acting in the same direction. The combined effects are greater than with either factor acting alone. The acidophiles still stand out prominently, however. The pituitaries of the limited diet estrone-injected chicks are smaller than in 20-day limited diet chicks or in 20-day normal diet chicks injected with 100 I. U. estrone (Table II).

Castrates Injected with Estrone

Twenty-day-old chicks castrated on the fifth day and injected daily with 100 I. U. estrone on the fifth to the ninth and the eleventh to the fourteenth days, showed no effects of castration on the pituitary. Some functional basophiles were present, but no more than were found in non-castrate uninjected controls. The size of the pituitaries of the experimental birds was 4843, approximately the same as in the controls which was 4715.

Pituitaries from 30-day-old chicks castrated on the fifth day and injected daily with 17.5 I. U. estrone from the tenth to the twenty-ninth days were decidedly castrate-like. The basophiles were much enlarged as were the nucleoli, while many of the acidophiles were partially degranulated and reduced in size. The basophiles showed no more signs of degranulation than in uninjected castrates. Degranulation of the acidophiles was no doubt primarily a castration effect and not due to the estrone injections.

The generally accepted interpretation of estrogen effects on the pituitary is that the estrogens inhibit the secretory activity of the anterior lobe and my observations favor this point of view. Severinghaus, however, says it is not possible to interpret the cytological data in this way if by secretory activity is meant the production and release of secretory substances. He believes there is a marked degranulation of both baso-

philes and acidophiles in estrogen-treated rats—also hypertrophy of the mitochondria. Estrogen then brings about a release of secretory materials. He is not so certain about the effects of estrogens upon the production of secretory substances. He argues, however, in favor of a stimulating effect upon production.

In the chick, as far as studied, the basophiles are restricted in their development and the acidophiles are not degranulated following estrogen injections. Instead of an enlargement of the entire anterior lobe, as Severinghaus and others report in the rat, there is actually a decrease in pituitary size in the chick. This decrease holds for dosages as low as five units daily for 26 days and for estrone injections into castrates, limited diet chicks, and controls. These size relations are consistent with the point of view that basophilic development is restricted and that acidophilic development is but little modified.

Brooding Hens

Brooding hens of known ages have been difficult to find, but Dr. Martin of Purdue University supplied two Barred Rocks, one of which was about 18 months and the other 30 months old. A larger sampling would have been better, but certain facts stand out clearly. In laying Barred Rock hens and in 25-day chicks of the same breed, the functional basophiles are very similar in size, shape, distribution and staining reactions to those found in White Leghorns. The cytoplasm stains a light green; the nucleus is large and stains similarly to the cytoplasm; and there are one or two spherical nucleoli. In the brooding hens the large basophiles have been reduced in number and have been replaced by much smaller cells, the nucleus of which is pycnotic and stains uniformly throughout except for the slightly darker nucleoli. In a comparatively small number of these cells, with pycnotic nuclei, the cytoplasm is also pycnotic. Such cells are inactive basophiles reduced in size until they are not much larger than chromophobes. With the exception of the extreme posterior end, they are distributed throughout the gland, but are most numerous in the mid-region.

There is a possibility that the acidophiles in the anterior end (A2) are more numerous than at any other time during which they have been observed, but the evidence is too meagre for any definite statements. The acidophiles in the posterior end (A1), however, are no more numerous than in laying hens.

THE PITUITARY IN OLD AGE

In discussing changes described as aging, it should be remembered that aging effects are relative and variable. When do these effects begin?

Minot has shown that the growth rate of the rabbit embryo is high in early stages, and then rapidly diminishes. Is this an aging effect? Robbins and others (1928), in their book on growth, give data with respect to egg production in the domestic fowl. Hens one year old lay the largest number of eggs and thereafter the number gradually grows less. Does aging in the hen begin at two years or even earlier in the pituitary and sex glands? Ordinarily, in discussing old age, we do not include these early changes; nevertheless they cannot be ignored and no one can say just when old age begins.

With our increasing knowledge of the endocrine glands and the part they play in physiological processes, the question has arisen whether changes in the activities of these glands may not be primary causes of aging. This question applies particularly to the pituitary, which is the source of several hormones and which influences directly or indirectly other endocrine glands and many metabolic activities.

Unfortunately, our information about pituitaries of aging animals is rather limited and perhaps some of it is erroneous. Carlson (Cowdry, 1939) quotes Parsons and Cooper to the effect that neutrophiles (chromophobes) increase throughout life and that basophiles invade the intermediate and posterior lobes. Carlson also states, "This gland appears, anatomically and cytologically, remarkably stable, from the age of 20 on to 80." He concludes that there is no valid evidence for either hypo- or hyper-function of the pituitary in old age in man. Severinghaus (1937) finds an increase in the number of basophiles in women over sixty years of age and accounts for this change by assuming old females to be physiological castrates. This suggestion, however, would not account for changes in the male nor are we certain that there is complete cessation of sex hormone secretion after the menopause. In fact, menopause urine is high in FSH, but there may be some source other than the pituitary. Hellbaum (1935) studied the gonad-stimulating activity of pituitaries from seven old mares which were beyond the reproductive period. Five of these pituitaries induced greater responses, judging by ovarian weight, than did pituitaries from pregnant mares, non-pregnant mares, stallions, geldings, colts, and foetuses. Two of these mares, however, which appeared older than the others, induced only slight follicular enlargements, results similar to those obtained with pituitaries from geldings more than seventeen years of age.

Hellbaum reports in a footnote in this same paper that in human beings of extreme old age, who have been afflicted with disease, the FSH potency of the pituitary was decidedly low. Whether this condition was due to disease, old age, or a combination of the two factors, he did not discuss, but inferred that it was due to old age plus disease.

Schooley (1937) states that both acidophiles and basophiles cease to function in senile animals.

My own observations on the pituitaries of old chicks have been limited because of the lack of availability of old birds of known age. Fortunately, however, I was able to get two White Leghorn hens thirteen years old from Professor T. B. Clark of the Department of Animal Husbandry of the University of West Virginia and five cocks, varying in ages from five to nine years from Dr. H. D. Goodale of Mount Hope Farm. Three of these cocks were Inbred Leghorns and two were Rhode Island Reds. Two of the leghorns were six and one eight years old. One Rhode Island Red was nine years old, the other five. A fourth White Leghorn cock, six years old and originally from the Mount Hope Farm, was obtained from Professor Martin of Purdue University. Professor Martin also furnished me with White Leghorn hens one, two, and three years old. Additional old fowls of unknown ages were obtained from the local poultry house.

While there is some variability, as expected, the pituitary changes in old fowls are consistent. The changes are gradual and all glands and all cells are not affected at the same time nor to the same degree.

In young birds the mitochondria in the basophiles are granules, varying in size and usually distributed at random through the cytoplasm. With fuchsin they stain uniformly a purplish red. The number is variable and seems to be greatest in older castrates. The granules may become vesicular and in older birds the vesicles may fuse to form larger vesicles, some of which are irregular in outline while others are spherical (Figs. 15 and 17, Plate II). There may be several of these larger fusion masses or only one.

With the acid fuchsin method of staining, the central areas of the masses are golden yellow while the peripheries are dark red. In osmicated material the peripheral region becomes black, while the central area looks much as it does in stained preparations. These mitochondrial spheres continue to enlarge. They not only may fill the cell (Fig. 11, Plate II), but the cell may eventually break down, leaving the spheres to occupy intercellular spaces. Since many basophiles may be affected, the golden masses present a striking picture in contrast to the background of the methyl-green stain. Figure 5, Plate I is an outline sketch which shows how numerous these spheres may be.

In speaking of the large spheres as vesicular mitochondria, I do not mean to imply that they are unmodified mitochondria. Just what mitochondria are from a chemical point of view I do not know, but they are probably combinations of several chemical substances including proteins

and lipids. Perhaps the principal change is due to the gradual infiltration of lipoidal substances.

Not all basophiles, however, become modified as described. Others, without undergoing such modifications, shrink in size and become pyknotic. In very old birds such as the two 13-year-old White Leghorn hens and the 9-year-old Rhode Island Red cock, it is doubtful whether any of the basophiles could have been functional. The entire glands were degenerate structures. In the 5-year-old Rhode Island Red and the 6-year-old Leghorns, a number of basophiles were certainly still functional but in the 8-year-old Leghorn a smaller number were functional.

While I have for the moment concluded that the starting point of the large spheres is a fusion of the vesicular mitochondria, I wish to point out that the tiny vesicles might possibly arise *de novo* independently of the mitochondria and that if they did, it would be practically impossible to distinguish between the two if they both reacted to the stain in the same way. It should be remembered, of course, that vesicular mitochondria are common in many animals and that their fusion is not uncommon. The most notable example of fusion is found in the formation of the Nebenkern of spermatids.

The formation of vesicular mitochondria and their fusion is also found in capons. There the evidence is even more convincing that vesicular mitochondria do actually fuse.

The acidophiles are very much reduced in number in all old birds, but they never completely disappear. Whether they are still functional cannot be stated from the cytological evidence alone. The cells, however, are much smaller than the large functional acidophiles of younger birds and the granules stain a darker purplish red.

In some of the acidophiles (A1) of all old fowls studied a spherical vesicle is found in the cytoplasm. See Fig. 17, Plate III for size and position. It does not stain but has a slightly yellowish or golden tinge. Whatever the substance is, it is not dissolved by the cytological reagents used. At first I thought it might be formed by a fusion of the mitochondrial granules much as found in the basophiles, but the vesicle and mitochondria are present at the same time in the same cell. On the other hand, the vesicle may arise by some modification of the Golgi substance but there is no direct evidence for such an origin. The Golgi substance in osmicated material is similar to that found in acidophiles of younger fowls. Origin *de novo* is a third possibility. The percentage of cells having this body was not determined, but as age progresses, the number increases.

A vesicle similar to that observed in the fowl is also found in the acidophiles of an old guinea-hen. Here the body is larger in comparison

to cell size, more conspicuous, and it seems there is one in every acidophile.

One of the first indications of age in the pituitary of fowls is the formation of acini (Fig. 6, Plate I), first at the posterior end of the gland, then near the periphery of the ventral surface, and finally at the anterior end. The juxta-neural and central regions are least affected. These acini are filled with material, perhaps colloidal in nature, but there is no direct evidence that it is secreted by the limiting cells. In fact, the cells are not granular and do not have the appearance of either actively secreting basophiles or acidophiles. Acini first appear in White Leghorn hens between two and three years old.

Another change common to pituitaries of old fowls is the presence of pigment granules. In extremely old birds most cells which have not already become highly degenerate, particularly those at the anterior end, are affected and the cytoplasm becomes filled with them. The granules are present in White Leghorn hens as early as two years of age.

Finally the chromophobes become reduced until only a few remain. The entire gland from a cellular point of view is a highly degenerate structure. It cannot be functional. All changes enumerated are gradual and it is only in very old birds that complete degeneration is found.

Gonads of the six old cocks were studied as well as pituitaries. The two 6-year-old Leghorns had large testes and sperm formation was actively taking place in all cords. In the third 6-year-old White Leghorn one testis was only half the size of the other and sperm formation was reduced in each. Both testes of the 8-year-old Inbred Leghorn were only about half size and sperm formation was very much reduced. Functional basophiles were present in all four pituitaries but the number was much less in the pituitary from the 8-year-old bird. Sperm formation in the 5-year-old Rhode Island Red was more reduced than in the 6-year-old Leghorns, in fact, even more so than in the 8-year-old Leghorn. In the 9-year-old Rhode Island Red, there were no indications of sperm formation and the cords had largely disappeared. A mass of cells with remnants of cord walls was all that remained. The pituitary of this bird probably had no functional basophiles. It seems clear that aging had progressed more rapidly in the Rhode Island Reds than in the leghorns—a varietal difference in all probability. The close correlation between the development of functional basophiles on the one hand and sperm formation and degeneration of the spermatogenic cords on the other is no mere coincidence. Changes within the testes are evidently dependent directly upon the activity or lack of activity of the secretory cells of the pituitary, and of the cells the basophiles probably play the more important rôle.

Pituitaries from White Leghorn hens one, two and three years of age were also studied. Acini and pigment granules were present at two and one-half and three and one-half years, but not at one year.

By way of summary it may be stated that pituitary changes in aging fowls are as follows:

1. Formation of acini, beginning as early as two and one-half years in White Leghorn hens.
2. Fusion of mitochondria and hypertrophy of the mitochondrial material in many of the basophiles.
3. Absence in old age of all functional basophiles. This is true for the two hens 13 years old and probably true for the 9-year-old Rhode Island Red cock.
4. Only a few granular acidophiles are present in all old hens and it is doubtful whether they can be functional. The same is true for old cocks. Younger cocks have not been studied.
5. Pigment granules are abundant in pituitaries of some old birds; less so in others.
6. Gradual disappearance of all, or practically all chromophobes.
7. Near the end comes degeneration of all cells. In cells on the way toward degeneration nuclei and cytoplasm are pycnotic.

These observations are at variance with the conclusions of Cooper and Parsons that the neutrophiles increase in number in old age, and with Carlson's conclusion that the pituitary in man from ages twenty to eighty years remains stable. Neither do these observations support Severinghaus' conclusion that the basophiles increase with age. They may increase in the fowl up to a certain point but after that, degenerative changes set in. Very definitely the evidence in old fowls points to pituitary changes in which all activity gradually diminishes and finally ceases. The gonad changes, in so far as sperm formation and cord structure are concerned, parallel closely the pituitary changes. Similar parallel changes must also occur in the ovaries of hens where egg production gradually grows less after the first year.

The data presented, however, give no conclusive evidence whether the pituitary changes are primary causal factors of aging processes in other parts of the body or whether they merely parallel other changes.

CONCLUSIONS

1. Cytological observations are in conformity with experimental data.
2. Functional basophiles are present in both male and female White Leghorn chicks at ten days of age. In Barred Rocks and Rhode Island Reds these cells do not function until after twenty days of age.

3. Acidophiles and basophiles arise from chromophobes and may regress, but whether they return to undifferentiated chromophobes from which either type of chromophile may arise is still questionable.

4. The evidence indicates there are two kinds of acidophiles.

5. Basophiles and acidophiles cannot be differentiated in early transitional stages on the appearance of the Golgi substance.

6. There is no division of the anterior pituitary in early embryonic development into an anterior and posterior lobe.

7. Limited diet as well as unfavorable conditions in general restricts the development of basophiles but has no adverse effects upon the acidophiles.

8. Castrate pituitaries are characterized by an unusual growth of the basophiles and a regression of the acidophiles.

9. Changes in the pituitary brought about by castration may be retarded by injections of estrogens and androgens.

10. In normal untreated chicks both estrogens and androgens retard the development of basophiles but have little or no effect upon the acidophiles.

11. When estrogens are injected into limited diet chicks, basophilic development seems completely blocked.

12. Acidophiles remain well developed in limited diet and estrone-injected chicks. Under both these conditions the intestine and liver are enlarged. These facts lend support to the suggestion that the acidophiles secrete prolactin or a growth-promoting substance affecting the intestine and liver.

13. In brooding Barred Rock hens many of the basophiles are much reduced in size and the nuclei are pycnotic. The A2 cells may be in a state of increased activity.

14. Aging effects begin between two and three years in the pituitaries of White Leghorn hens. The effects are progressive and by thirteen years the gland is probably not functional. In the nine-year-old Rhode Island Red cock the pituitary was not functional and sperm formation had ceased entirely.

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THE VERTICAL MIGRATION OF THE COPEPOD ACARTIA TONSA UNDER CONTROLLED ILLUMINATION

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INTRODUCTION

Vast numbers of plankton organisms are known to rise toward the surface of the water at night and sink downward during the day. This vertical migration was first studied by the great oceanic expeditions of the last century, particularly that of the "Challenger" in 1872 (Murray and Hjort, 1912). The first laboratory investigations were those of Groom and Loeb (1890) and Parker (1902). The earlier work has been extensively reviewed by Russell (1927) and Kikuchi (1930).

It was soon apparent that light was the main environmental factor involved, but many diverse conclusions were made as to its mode of action. These may be summarized as follows:

(1) *Phototropism overcoming geotropism*.—Parker (1902) found that *Labidocera aestiva* rose at night because of its negative geotropism, but sank during the day because this was overcome by its negative phototropism.

(2) *Light changing the sign of geotropism*.—*Cyclops albidus* was found to be geopositive in the light and geonegative in the dark (Esterly, 1907). Field observations on *Cyclops* by Worthington (1931) and Southern and Gardiner (1932) support this view.

(3) *Optimum light intensity*.—Rose (1925) placed various copepods in a horizontal tube with a graded light intensity, and found that the animals gathered in an intermediate zone. Field observations indicate that *Calanus* (Russell, 1926, 1928, 1934), *Metridia* (Clarke, 1933, 1934) and other forms follow a zone of optimum intensity.

(4) *Relative optimum intensity*.—*Daphnia* was found by Clarke (1930) to have a "primary" negative phototropism, which is strengthened by an increase of light intensity. When the intensity is reduced, the animal acquires a "secondary" positive phototropism and moves toward the light as long as it is being reduced. But if the intensity is held constant, the primary sign returns, and the animal moves away from the light. There is only a relative optimum here, the animal becoming adapted to the intensity existing at the moment. Such a mechanism is suggested by field observations of *Acartia clausi* (Johnson, 1938).

Other experiments have led to diverse conclusions. Johnson and Rayment (1939) found that *Centropages typicus* moved towards the light when the intensity is either increased or decreased. Downward movements during the night, when light intensity is constant, have been reported by several field workers (e.g., Waterman et al., 1939). These authors suggest that light merely keeps in phase internal physiological cycles which regulate the migration of the organisms. Esterly (1917b) found that *Acartia tonsa* kept in constant darkness moved upward on two successive evenings, suggesting the influence of an internal rhythm.

In none of these studies has vertical migration actually been reproduced. Groom and Loeb (1890) watched *Balanus* nauplii performing their diurnal migrations in a glass of water on the window, while Parker (1902) noted the migration of *Labidocera* in a floating jar outside the laboratory. But aside from these early observations, there are no reports of diurnal migrations being performed under controlled conditions. Many of the conflicting results may have been caused by subjecting the animals to laboratory situations which would never be encountered in nature. Thus Loeb (1908) concluded that vertical migration is caused by the difference in temperature between warm waters near the surface and cool deep waters. This obviously cannot apply to migration in those lakes where no temperature gradient occurs (Worthington, 1931). The observation of Clarke (1930) that the phototropic sign of *Daphnia* is reversed by changing light intensity, cannot apply to the downward migration of this form beginning at midnight (Southern and Gardiner, 1932). It was therefore decided to inquire if vertical migration could be reproduced under conditions approximating natural ones as closely as is possible in the laboratory. If so, it would then be possible to alter various factors in order to find the controlling ones.

APPARATUS AND PROCEDURE

Animals collected by towing an open net were brought into the laboratory and placed in tall glass cylinders exposed to window light. The cylinders were placed in a large tank filled with water to permit control of the temperature. Observations were made by removing the cylinders from the tank and noting the vertical distribution of the animals.

Specimens of the copepod *Acartia tonsa* Dana were obtained by towing an open net for five minutes at a depth of 3 meters at the head of Woods Hole Harbor. The animals were collected in a small bottle towed at the end of the net; to avoid overcrowding they were transferred to a larger bottle as soon as the net was hauled aboard. The catch was

immediately brought into the laboratory and poured into finger bowls. Individual *Acartia tonsa* were identified under a dissecting microscope, picked up in a pipette, and placed 20 each in six glass cylinders, 48.5×4.5 cm., filled with sea water to a height of 43–44 cm. The cylinders were placed in a black-walled tank, $46 \times 66 \times 45$ cm., open at the top, and placed in front of a window. The tank was filled to the water line in the cylinders with fresh water, which was kept at 15° by a thermostat attached to a refrigerating unit. Surface temperature in the harbor at the time of the hauls was $18\text{--}21^\circ$ C.

The animals were fed with the diatom *Nitzschia closterium*, found by Clarke and Gellis (1935) to be a favorable diet for copepods. The diatoms were cultured by the method of Ketchum and Redfield (1938). A few drops of this culture were added to each cylinder every morning. Varying the time of feeding did not affect the behavior of the copepods.

Observations were made by counting the number of animals swimming in each vertical third of each cylinder. Since it was not possible to see animals lying on the bottoms of the cylinders, they were computed by subtracting the total number of animals swimming at any count from the highest total obtained in subsequent counts in that cylinder during the day. Thus, if six animals were observed swimming in cylinder II at 8:00 A.M., while ten animals were found swimming in the highest subsequent count of the day, it was assumed that four animals were resting on the bottom at 8:00 A.M. Dead animals are thereby eliminated, since they appear in no subsequent count of animals swimming. From this data, the percentage of animals in the top third of the cylinders was computed for each observation.

The cylinder to be counted was taken out of the tank and placed on a nearby shelf. Observations in the darkroom were made by turning on a 25-watt red lamp for one to two minutes while the animals were counted. This brief exposure to light did not affect their relative position. The remaining cylinders were covered during this time. The chief source of error in this procedure lies in the necessity of removing the cylinders from the tank for counting. Any such error is probably constant under the same light conditions, but may affect the results when different conditions are being compared.

OBSERVATIONS

Normal Behavior

The cylinders were first placed in front of an unobstructed south window, so arranged that they were never exposed to direct sunlight. Photometric observations showed that the light reaching the tank was

of approximately the same intensity as that at a depth of 10–20 meters in Woods Hole Harbor (Oster and Clarke, 1935). Normal vertical migration was carried on under these conditions for at least eight successive days. The animals moved up rapidly immediately following sunset, and then more slowly. The downward movement commenced at midnight, and proceeded slowly until dawn, when it became more rapid. The daytime level remained relatively constant (Fig. 1). The only field observations of the diurnal movements of *A. tonsa* are those made at La Jolla, California, by Esterly (1928). When the two curves

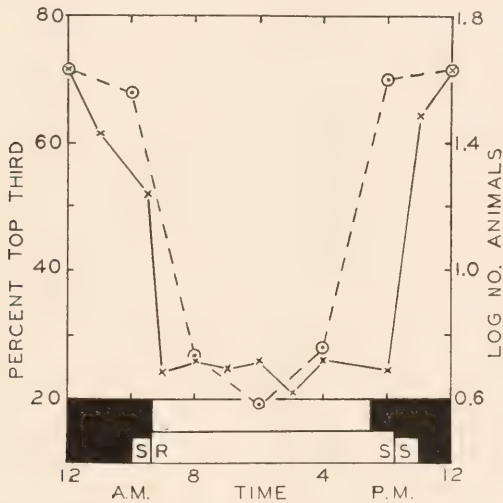


FIG. 1. Vertical distribution of *Acartia tonsa* in laboratory (solid line). Ordinate, percentage of animals in top third of cylinders; abscissa, time of day. Distribution in field (dotted line) from Esterly, 1928. Ordinate, mean logarithm of number of animals per haul; abscissa, time of day. SR represents sunrise, SS sunset. Upper bar for La Jolla, lower for Woods Hole. The shorter day in California seems to be correlated with a shorter downward movement.

are superimposed, the similarity of their shape indicates that the laboratory behavior is representative of that in the field.

An attempt was made to reproduce this behavior under more carefully controlled conditions. Room light was excluded by placing a tarpaper shelter over the tank. The cylinders were illuminated with daylight fluorescent lamps, chosen because their spectral energy distribution is approximately the same as that of daylight (General Electric Co., 1939). An opal glass diffusion screen was placed over these lamps to imitate the broad source area of window light. Four 24" lamps were used, covered with a 14 × 24" opal screen. To reproduce the

angle at which window light entered, the lamps were placed 60 cm. above the tank and 80 cm. to the side. The light now entered the cylinders at an average angle of 36° from the horizontal. Normal vertical migration continues under these conditions. The animals stay at the bottom of the cylinders during the day when the lights are on, and rise to the top at night when the lights are off (Table I).

TABLE I

Fluorescent light. Average of 60 A. tonsa at 15° C. for 48 hours; July 28-30, 1941.
Observations in darkness are italicized.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
6 A.M.	52.2	6 P.M.	9.55
8	13.4	8	7.97
10	3.50	10	62.2
2 P.M.	9.45	12 Midnight	52.1

Diurnal Rhythms

If this vertical migration is controlled by an internal rhythm, the normal movements should continue in constant darkness. Although the animals began their normal descent on the first morning of darkness, they did not leave the upper portion of the cylinders (Fig. 2). While no migration appears under these conditions, the normal behavior may be controlled by the coincidence of light and an internal rhythm set for the normal alteration of night and day. That this is not so is seen by the fact that the migration can be reversed by illuminating the animals at night and keeping them in the dark by day (Table II). It is significant, however, that the upward movement during the first reversed day is appreciably less than during the second. This behavior, together

TABLE II

Reversed day and night. Average of 120 A. tonsa at 15° C.

Observations in darkness are italicized.

July 31-August 1, 1941: Normal fluorescent light.

August 1-4: Reversed day and night.

Date	Percentage of Animals in Top Third of Cylinders					
	2 A.M.	8 A.M.	10 A.M.	2 P.M.	8 P.M.	10 P.M.
July 31					10.5	51.1
Aug. 1		61.6	0.81	21.6	10.8	14.3
2		14.3	37.1	44.1	42.5	12.1
3	18.2	15.2	62.6	51.8	64.3	51.8
4		33.4				

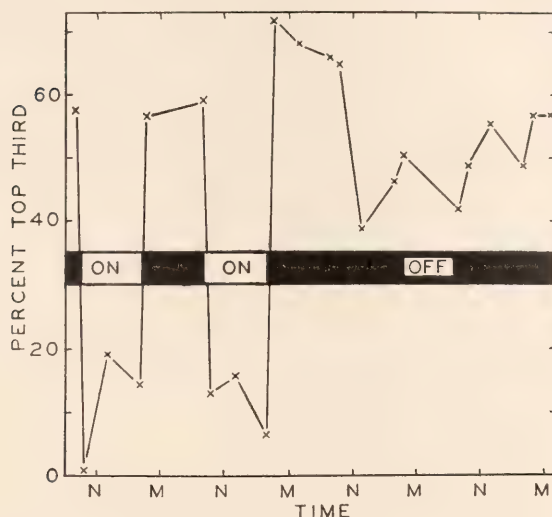


FIG. 2. Vertical distribution of *A. tonsa* in daylight fluorescent light and in constant darkness. Ordinate, percentage of animals in top third of cylinders; abscissa, time of day. N = noon; M, midnight.

with the downward movement during the first morning of constant darkness, indicates that traces of a metabolic rhythm, once set up, persist under changed conditions for one day. Although light alone seems to control the behavior of the animals under these conditions, it is possible that it is effective only if it operates in a 24-hour cycle. This is not so, however, since a 4-hour "day" can be induced (Table III).

TABLE III

Four-hour day. Average of 120 *A. tonsa* at 15° C.; August 4, 1941—fluorescent light. Observations in darkness are italicized.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
10 A.M.	0.40	1:00 P.M.	<i>40.0</i>
11	<i>28.0</i>	1:15	0.40
12 Noon	<i>48.0</i>		

Changes in Illumination

The lighting conditions were now altered in order to find the specific factors controlling the migration of *A. tonsa*. No difference in behavior appeared when the opal glass screen was removed, showing that the area of the light source was of no significance (cf. Fig. 2: the first day is without the screen, the second with it). Spectral energy distribution

was found not to be a controlling factor, since incandescent bulbs were substituted for fluorescent lamps without effect. Bulbs of 25, 60 and 300 watts were used without changing the normal behavior, showing that light intensity within this range is not a factor in migration (Fig. 4).

An entirely different situation prevailed when the lamps were moved directly above the tank. Migration was now reversed, the animals mov-

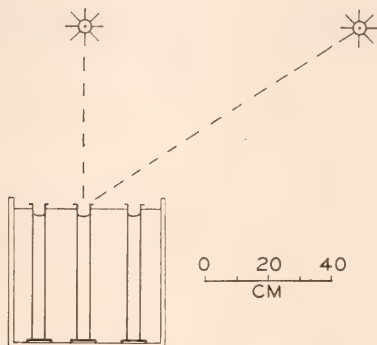


FIG. 3. Arrangement of oblique and overhead lights. Distances in centimeters.

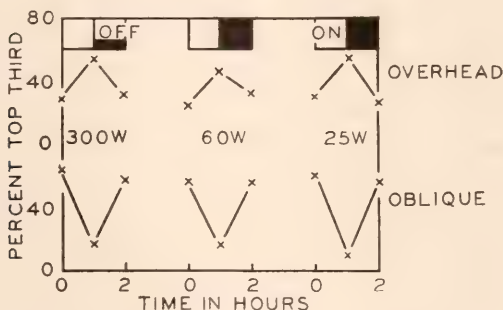


FIG. 4. Vertical distribution of *A. tonsa* in incandescent light. Top, lamps 60 cm. above tank; bottom, same, but 80 cm. to side. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours.

ing up in the light and down in the dark (Fig. 4). If the overhead lights are kept on for 24 hours, the copepods stay up throughout this period, although animals in oblique light for 24 hours stay down (Fig. 5). The animals move *up* in overhead light, or in darkness following oblique light; they move *down* in oblique light, or in darkness following overhead light. The downward movement when the overhead light is turned off lasts for only an hour. After this time the normal upward movement in darkness begins (Fig. 6). This upward movement con-

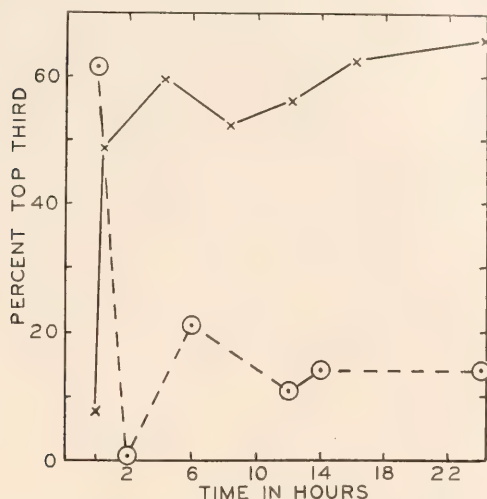


FIG. 5. Vertical distribution of *A. tonsa* in daylight fluorescent light. Solid line, lamps 60 cm. above tank; dotted line, same, but 80 cm. to side. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours.

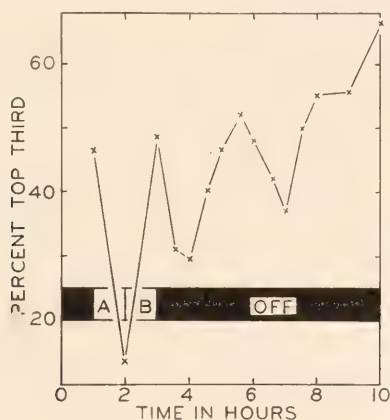


FIG. 6. Vertical distribution of *A. tonsa* under various light conditions. Ordinate, percentage of animals in top third of cylinders; abscissa, time in hours. A, Daylight fluorescent oblique; B, 25-watt overhead.

tinues for $1\frac{1}{2}$ hours, to be followed by another downward movement, which in turn is followed in $1\frac{1}{2}$ hours by an upward movement. If the animals have been kept up for 12 hours with overhead light, they will stay down in the dark for the next 12 hours.

A simple but dramatic presentation of these results can be made by leaving a cylinder containing *A. tonsa* in the dark for an hour. If a

weak red light is now turned on for a few moments, the animals may be seen slowly swimming about, the greater number at the top of the cylinder. If a white light, level with the center of the cylinder but several feet away, is now turned on, a remarkable change occurs in the behavior of the animals. First one and then another stops swimming and sinks slowly downward, head up, antennae outspread. Occasionally an individual swims about for a moment, but it soon stops and continues sinking downward. In ten minutes almost all the animals will be at the bottom of the cylinder. If the light is now placed on top of the tube, the animals swim rapidly upward. Some individuals, seemingly unable to get close enough to the light, hurl themselves time after time against the surface of the water.

Movements of Individuals

It has hitherto been impossible to determine whether vertical migration consists of mass movements of entire populations, or of a general trend of diverse individual movements. Thus Fig. 1 shows that about 20 per cent of the animals are on the surface during the day, and 70 per cent at night. Does this mean that only 50 per cent of the animals actually migrate, or that all the animals spend 20 per cent of their time on the surface during the day, and 70 per cent at night? To answer this question, 42 individuals were tested in oblique light, in overhead light,

TABLE IV
Individual movements of 42 A. tonsa at 15° C.; August 18-23, 1941.

Light Conditions	Time	Animals in Top Third of Cylinders	Average Movement of 42 Individuals*
	<i>hours</i>	<i>per cent</i>	
Dark	1	60.0	
	2	58.4	1.9
	3	77.8	1.8
	4	63.8	1.5
Fluorescent Oblique	5	23.3	2.0
	6	23.3	2.2
	7	27.8	1.6
	8	27.8	1.6
Dark	9	63.4	1.6
	10	66.6	1.9
	11	58.3	1.7
	12	79.2	1.2
25w Overhead	13	72.2	1.1
	14	58.3	1.2

* Each unit represents one-sixth of cylinder, or 7 to 7.5 cm.

and in the dark. The cylinders were divided into sixths numbered from top to bottom. The average distance moved per hour was measured in units representing one-sixth of a cylinder (Table IV, Fig. 7*A*). The average behavior of the entire group gives the same results as before, but the individuals are seen to be continually moving. Each animal traverses about one-third of a cylinder (14–15 cm.) every hour. Maximum movement occurs two hours after the lighting is changed. Too

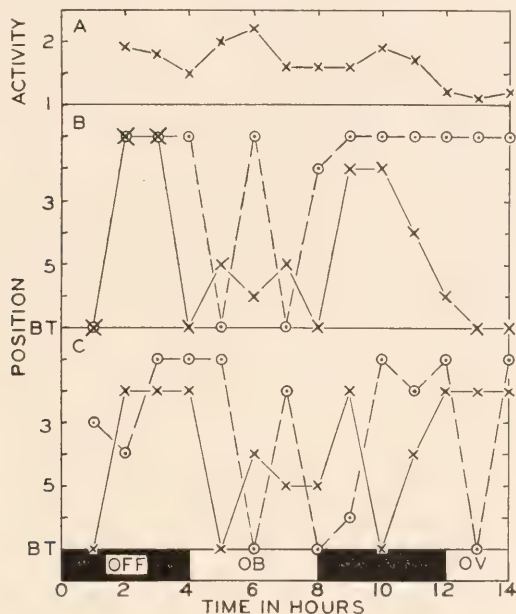


FIG. 7. *A*, average movements of 42 individuals. Ordinate, activity in units representing one-sixth of a cylinder; abscissa, time in hours. *B*, Positions of two males. *C*, Positions of 2 females. Ordinate, position of animals in sixths of cylinders, numbered from top to bottom; abscissa, time in hours; *Bt* indicates resting on bottom; *OB* indicates daylight fluorescent, oblique; *OV*, 25-watt, overhead.

few males were present to permit a statistical comparison of the sexes, but no significant differences are found between the records of individual males and females (Fig. 7). Because of the greater distances covered, the proportion of these scattered movements to the entire migration may not be as great in nature as in the laboratory. These diverse movements suggest why field observations do not show that all of the animals are right on the surface at night, and down on the bottom during the day.

DISCUSSION

It might appear that the difference between the behavior of the animals in overhead and in oblique light is caused by the intensity of the light, since the lamp is closer to the cylinders in the overhead than in the oblique position. But measurements of light intensity show that this factor must be eliminated, since the intensities of the various sources of oblique and overhead light overlap (Table V). The only remaining difference is in the positions of the lamps.

TABLE V

Light intensity measured by "Photox" cell with $\frac{1}{2}$ " diaphragm.

Light Source	Position of Bulb	
	Oblique	Overhead
Window	2.8 m.a.	
Incandescent bulb		
25-watt	0.8	1.7 m.a.
60-watt	1.5	3.8
300-watt	5.2	14.0
Fluorescent lamps		
with screen	1.0	
without	2.5	

It is believed that the control of this behavior lies in the directional character of the illumination. The overhead light appeared to shine directly down the cylinders with little deflection. The oblique light, on the other hand, entering at an average angle of 36° from the horizontal, appeared to be reflected from the walls of the tank and cylinders so as to provide a more diffuse illumination (Fig. 3). Additional evidence is provided by tropism experiments in a horizontal glass trough. This trough, which could be illuminated by an incandescent bulb at either end, was surrounded by water to minimize reflection from the side. Under this highly directional illumination, *A. tonsa* always moved toward the light. By turning on first the lamp at one end and then that at the other, it was possible to keep an individual moving back and forth from end to end indefinitely. But the distribution of light in natural waters, according to recent measurements, does not show this highly concentrated character. Whitney (1941) found that the light intensity at a depth of 3 meters in Woods Hole Harbor was distributed as follows:

overhead 12 units
 towards sun 16 units
 horizontally 2 units
 from below 1 unit

Although similar measurements could not be made in the cylinders, the oblique light appeared to provide a comparable situation. Overhead light, or the light in the horizontal trough, seemed to be of a much more concentrated character. It is believed that this difference in the concentration of the light is the only factor that can account for the difference in behavior.

This view is supported by several reports in the literature of animals which are positive to direct light, but which move down in diffuse light and up in the dark. Such behavior has been found in the larva of *Corsethra plumicornis* (Harper, 1907), in *Branchipus serratus* (McGinnis, 1911), *Daphnia pulex* (Dice, 1914), and *Sagitta bipunctata* (Esterly, 1919). Two of these three relations have been described for *Cyclops albidus* (Esterly, 1907), for the nauplii of *Balanus perforatus* (Ewald, 1912), for *Paramoecium* and the larvae of the echinoid *Diadema setosum* (Fox, 1925), and for six Crustacea (Kikuchi, 1938).

In diffuse light, all parts of the photoreceptor will receive illumination of equal intensity. In direct light, on the other hand, that part of the receptor facing the source will be strongly illuminated, while much less light will come from the side. There is no need for the light to be perfectly direct or diffuse, since a threshold ratio of front light to side light may determine the behavior.

We may then conclude that:

(1) *Acartia tonsa* sinks downward in light which is not highly directional. This behavior, together with its upward movement in darkness, is believed to account for the normal vertical migration of this form.

(2) *A. tonsa* moves toward a source of highly directional light. Such illumination is not likely to be encountered in nature.

This theory will explain the paradox of an animal which moves toward the light in the laboratory, but away from it in the field. It seems to account for the fact that positively phototropic plankton do not come to the surface of the sea during the day, and also, that positively phototropic insects do not fly up to the sun.

If light alone controls the movements of these animals, we could not account for the oscillating movements in darkness following overhead light (Fig. 6). The movement toward the light, however, may result in fatigue, and the animal will then sink down. On recovery, the animal swims up, to sink down again when fatigued. Such oscillations may explain downward movements beginning at midnight, reported from both field and laboratory (Fig. 1), as well as the upward movements in constant darkness observed by Esterly (1917b).

There is no justification for applying the results of these experiments to vertical migration in general. Thus, while *Acartia tonsa* continues its response to light for at least 48 hours, another species of the same genus, *A. clausi*, becomes indifferent after 30 minutes (Johnson, 1938). Experiments with *Centropages hamatus*, a copepod found together with *A. tonsa*, show that it performs no migration when exposed to window light. An average of 52.1 per cent of the animals remained in the top third of the cylinders throughout the day and night (Table VI). Every field observation shows that different genera and species migrate at different times and rates. Differences between broods of the same

TABLE VI

Centropages hamatus. Average of 120 animals at 15° C. for 24 hours.
Window light, July 11-12, 1941.

Time	Percentage of Animals in Top Third of Cylinders	Time	Percentage of Animals in Top Third of Cylinders
2 A.M.	56.4	2 P.M.	53.2
4	56.2	4	66.7
6	39.3	6	56.1
8	44.9	8	49.0
10	48.3	10	51.8
12 Noon	59.4	12 Midnight	44.6

Average time of sunrise, 5:16 A.M.; sunset, 8:21 P.M.

species have been found (Russell, 1928), and even between sexes of the same brood. Thus Langford (1938) finds that male *Diaptomus* moves down at the same time that the female is moving up. It is apparent that each plankton form must be investigated separately. While future investigation may show that all vertical migration is due to a single cause, it is equally probable from the data on hand that many different factors are responsible.

It is hoped to continue these experiments with other species of plankton. Isolated observation of the effect of various factors on behavior has no necessary relation to conditions in the field. If vertical migration is first reproduced in the laboratory, one can be reasonably sure that field conditions are being represented. Only then should various factors be altered in order to find the controlling ones.

SUMMARY

1. The natural diurnal vertical migration of the copepod *Acartia tonsa* can be reproduced in the laboratory. Animals kept in tall glass cylinders exposed to window light move up at night and down during the day.

2. Incandescent or fluorescent lights can be substituted for daylight without affecting this behavior, provided that the illumination is oblique to the axis of the cylinders. Migration does not depend upon the area of the source, spectral energy distribution, or total intensity of the light.

3. When illuminated obliquely to the axis of the cylinder, *A. tonsa* sinks downward. This behavior, which is followed by an upward movement in darkness, is believed to account for the normal migration of this form.

4. When the illumination is parallel to the axis of either a vertical or a horizontal tube, *A. tonsa* swims toward the light. Such highly directional illumination is not likely to be encountered in nature, and this behavior is believed to be a laboratory artifact.

5. Observation of individuals shows that the migration consists of a general trend of scattered movements. No difference was found between the sexes.

6. No evidence was found for a diurnal rhythm other than that caused by the normal alternation of day and night. Migration ceases under constant conditions; it can be reversed by illuminating the animals at night and keeping them in the dark by day; and the normal 24-hour behavior can be compressed into four hours.

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ULTRAVIOLET LIGHT AND THE DEVELOPMENT OF FUCUS EGGS AS AFFECTED BY AUXIN AND pH¹

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INTRODUCTION

It has been shown earlier (Whitaker, 1941) that unilateral irradiation with suitable doses of a number of wave-lengths of ultraviolet light (2345, 2482, 2537, 2654, 2804, 3130, 3660 Å) causes *Fucus* eggs to develop rhizoids on their non-irradiated halves. In the case of the wave-length 2804 Å, it was further shown that the responsiveness of the eggs increases gradually after fertilization, reaching a maximum in about 7 hours, and that the response of a population of eggs is proportional, over a wide range, to the logarithm of the dosage. Sufficiently strong doses inhibit rhizoid formation altogether. In an earlier paper in which these results were reported (Whitaker, 1941), it was suggested that inactivation or destruction of growth substance (auxin) in the eggs might be the principal means of action of the shorter wave-lengths of ultraviolet. This would suppose that moderate doses determine polarity by differentially destroying auxin on one side of the egg, and that strong doses might prevent rhizoid formation by destroying auxin throughout the egg. If this were so, it might be expected that adding auxin (β -indole acetic acid) to the medium after irradiation would tend to counteract the effects of ultraviolet.² The present experiments make this test. Experiments are carried out in sea water acidified to pH 6.0 (which approaches the limit of acid tolerance of the *Fucus* egg) as well as in normal sea water at pH 8.0 because the penetration of β -indole acetic acid into the eggs is favored by reduced dissociation in acid medium. Auxin is also more effective physiologically under acid conditions, i.e. in the molecular form (Bonner, 1934).

The effect of pH of the medium on the response of the eggs to ultraviolet was also determined to provide further information about the reactions in the protoplasm caused by the ultraviolet.

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² This might be expected even if a uniform application of auxin did not abolish a gradient of concentration in an egg irradiated with a moderate dose from one side, if enough auxin is supplied so that the concentration at the irradiated surface reaches or passes an optimum.

The shorter wave-lengths of ultraviolet are heavily absorbed by the protoplasm of the *Fucus* egg (Whitaker, 1941) so that the immediate effect of the ultraviolet must be greatest in the cortical region on the irradiated side of the egg. This raises a question that is broader than the rôle of auxin. Do the rhizoids form on the non-irradiated halves of the eggs because the ultraviolet so damages the cortex upon which it falls that rhizoid formation becomes impossible? If so, the rhizoids would have to form, if at all, on the non-irradiated halves of the eggs, as observed. One series of experiments is designed to answer this question.

MATERIAL AND METHOD

Fucus furcatus was collected at Moss Beach and at Pescadero Point, California, from December 1940 to July 1941, inclusive. The gametes were obtained by methods already described, and fertilization was caused to take place at a determined time, ± 7 minutes. Filtered sea water (Sp. gr. 1.026–1.027) at pH 7.9–8.1 was used as the medium throughout, except where otherwise specifically noted. When sea water was acidified to pH 6.0, approximately one-tenth part of Sorensen's buffer was added and the mixture was aerated with a scintered glass nozzle until pH equilibrium became established. No consequential change in specific gravity resulted. The eggs were reared in a constant temperature room at $15 \pm \frac{1}{4}^{\circ}$ C., and were shielded from white light until the final results were observed.

The ultraviolet source was a gas tube mercury resonance lamp made by Westinghouse, and sold under the trade name Sterilamp. The lamp has a 10-inch tube made of Corex glass, which stops almost all of the λ 1800 Å band. The lamp produces practically no heat and more than 90 per cent of the radiant energy is of the wave-length 2537 Å. There are small amounts of λ 3130 and 3660 Å, and some bluish visible light. It has been shown earlier (Whitaker, 1941) that pure λ 2537 Å directs the position of rhizoid formation in *Fucus*, and also that λ 3130 and 3660 are relatively ineffectual compared with the shorter wave-lengths of ultraviolet. For present purposes, therefore, the effects of the radiation from the Sterilamp can be considered quite dominated by the effects of λ 2537 Å.

Four clear fused quartz culture vessels of a considerable degree of optical perfection were made by the Hanovia Chemical and Manufacturing Company. These vessels measured $50 \times 25 \times 15$ mm., and eggs were irradiated and reared in single or double rows on slabs of plate glass resting on the bottoms inside the quartz vessels. When there were two parallel rows of eggs, the second row was placed on a glass shelf

above and behind the first row so that no eggs were eclipsed. In all cases the eggs within the rows were at least 5 egg diameters apart, and the ultraviolet light passed through $0.9 \pm .1$ cm. of medium before reaching the eggs. The culture vessels were maintained on a leveled glass platform so that the eggs did not roll, and a microscope was arranged so that the eggs could be observed at any time with red light without touching the platform or vessels.

The unit of dosage adopted in this work represents the application to the eggs of 1 erg per square millimeter, without correction for scatter and absorption in passing through the vessel wall and 9 mm. of sea water. The scatter and absorption are minor, and one unit very nearly represents the application of 1 erg per square millimeter to the surfaces of the eggs. The intensity of the ultraviolet shorter than λ 3200 Å emitted by the Sterilamp was measured by means of a Hanovia Ultraviolet meter. The intensity of the lamp source under working conditions did not vary more than about 6 per cent. Its intensity was measured from time to time during an exposure, and the variation was compensated for by adjusting the duration of exposure to give the desired total dose.

The distance of the Sterilamp, and the length of lamp tube used, were varied to obtain great range of effective intensity. When the dosage was 1–100 units, the lamp was 4 feet from the eggs; 4 cm. of lamp tube was used and energy was supplied to the eggs at the rate of about 1 unit every 16 seconds. When the dosage was 20,000–50,000 units, the lamp was 6 inches from the eggs, and energy was supplied to the eggs at the rate of about 525 units per minute. Individual shutters were used to interrupt the ultraviolet to each vessel, and when the vessels were relatively near the lamp, parallel side baffles ran from either end of each vessel to the lamp tube so that the eggs did not receive ultraviolet from too great an angular span of directions. The beam of ultraviolet approached the vessels with a downward slant of about 3° to insure against eclipsing by the forward margin of the slab upon which the eggs rested.

When strong dosages (20,000–50,000 units) of ultraviolet were applied, development was greatly delayed. Therefore, after such strong irradiations the eggs were transferred on the glass slabs to glass vessels for rearing, in order to free the quartz vessels for irradiating eggs for new experiments. There may have been some tendency for the eggs to roll during transfer, and partly for this reason the direction of rhizoid formation was not counted in the experiments with strong dosages. The retardation and inhibition of development was measured in these cases.

RESULTS

The Relation between Dosage and Response

Experiments were designed to test the relation between dosage of λ 2537 Å and the response of populations of eggs, in the lower dosage range in which the position of rhizoid formation is determined but development is not impeded. The eggs had been fertilized 8 hours when the irradiation began.

The sensitivity of different samples of eggs varies somewhat, especially as the season progresses. It was therefore necessary to cover nearly the whole span of dosages in each experiment, so that balanced averages might be obtained when all experiments were brought together. Four culture vessels in each experiment were exposed to different dosages, and the average results of 11 experiments are shown graphically in Fig. 1. The points at 1, 2, 5, and 10 units are each based on 550–670 eggs, and the point at 50 units is based on 340 eggs. It is clear that the response of populations is proportional, over a wide range, to the logarithm of the dosage.

This curve (Fig. 1) gives a basis for selecting the dosage for tests of the effect of auxin and pH on the sensitivity of the eggs to ultraviolet. Since 50 per cent of the rhizoids in a population form away from any given side of the culture vessel under conditions of randomness, it is the excess above 50 per cent forming on the halves of the eggs away from the source of radiation that measures the effect of the radiation. Seventy-five per cent represents half of the maximal response to ultraviolet, and while the dosage required to cause this response will vary somewhat, interpolation on Fig. 1 indicates that 2.8 units is a good dosage to use.

Rhizoid Formation on the Surface Irradiated with Ultraviolet

The question was raised at the end of the Introduction whether the irradiated surface of the egg is so damaged by destructive wave-lengths of ultraviolet that rhizoid formation is impossible on this surface. If so, in determining the developmental axis the ultraviolet might merely be acting through general breakdown of a non-specific nature. To find out whether the irradiated surface is capable of rhizoid formation, eggs were first irradiated from one side with 50 and 100 units of λ 2537 Å, since these dosages are more than adequate to cause rhizoids to form on the non-irradiated sides of the eggs (see Fig. 1). The midpoint of the period of irradiation was at 8 hours after fertilization, which is still many hours before rhizoids form (Whitaker and Lowrance, 1936). Immediately after the irradiation with ultraviolet, an ordinary 75-watt

bulb at 1 meter distance was turned on so that the eggs in half of the vessels were irradiated with white light from the opposite side. The white light was left on continuously until results were observed at 26 hours after fertilization. The response of *Fucus* eggs to unilateral irradiation with white light is to form the rhizoids on the sides of the eggs away from the light (Whitaker and Lowrance, 1936). The directional demands of the ultraviolet and of the white light were therefore

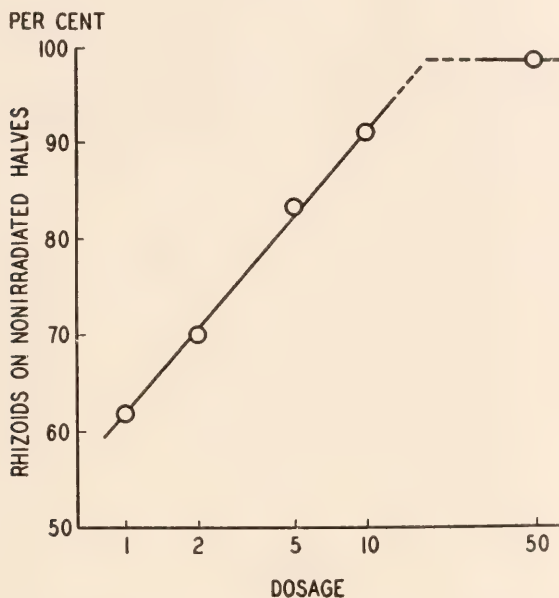


FIG. 1. The vertical axis indicates the percentage of eggs in populations that formed rhizoids on the halves away from the source of radiation after unilateral irradiation with λ 2537 Å. The horizontal axis shows the dosage on a logarithmic scale. One unit of dosage represents approximately 1 erg per square millimeter (see text).

opposed in these experiments, but the white light was left on much longer to dominate.

The results are shown in Table I. It is clear that the white light reverses the direction of rhizoid formation so that rhizoids form on the surfaces of the eggs that were irradiated with ultraviolet. This appears to indicate that the unilateral ultraviolet does not determine the position of rhizoid formation merely by causing extreme destruction and incapacitation of the irradiated surface of the egg. Some more specific effect is to be sought.

The Effect of Auxin on the Response to Low Dosages of Ultraviolet

A series of experiments was designed to test the effect of β -indole acetic acid (auxin) on the sensitivity of eggs to low dosages of λ 2537 Å that determine the polarity of some of the eggs in a population. Eggs were irradiated from one side with 2.8 units at 8 hours after fertilization. In each of 7 experiments two control vessels contained normal sea water throughout, while in two other vessels the medium was changed im-

TABLE I

Results of experiments in which eggs were irradiated from one side with 50 and 100 ergs per square millimeter of ultraviolet light (λ 2537 Å). In the second part of each experiment, the irradiation with ultraviolet was followed by a more prolonged irradiation with white light from the opposite sides of the eggs.

Exp.	Total No. Eggs	Treatment	Rhizoids on Halves of Eggs Exposed to Ultraviolet
			per cent
1	238	50 ergs per sq. mm. unilateral ultraviolet	6
	220	50 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
2	205	50 ergs per sq. mm. unilateral ultraviolet	2
	192	50 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
3	466	100 ergs per sq. mm. unilateral ultraviolet	4
	352	100 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100
4	209	100 ergs per sq. mm. unilateral ultraviolet	0.5
	176	100 ergs per sq. mm. unilateral ultraviolet followed by opposed white light	100

mediately after irradiation to sea water containing 150 mg. per liter of β -indole acetic acid at pH 8.0. A small amount of NaOH was added to maintain pH at 8.0. This concentration of β -indole acetic acid was the greatest that could be used at pH 8.0 without considerably delaying the development of the eggs. In most but not all of the experiments the eggs in the solution containing β -indole acetic acid gave a somewhat greater response. The overall averages for the 7 experiments, involving 1230 control eggs in sea water and 1200 eggs in the β -indole acetic acid-sea water solution, show that 64 per cent formed rhizoids on the halves

away from the ultraviolet in normal sea water, while 69 per cent did so in the sea water containing β -indole acetic acid. The difference is significant. It can be concluded, however, that the effect of adding β -indole acetic acid, even in amounts beginning to be toxic, is slight, and it does not tend to decrease the effect of the ultraviolet.

Another series of experiments was carried out in which the same concentration of β -indole acetic acid was added some time before irradiation. Its presence greatly decreased the response of the eggs, but absorption measurements showed that this could be explained by the very great extinction of the ultraviolet in passing through 9 mm. of the solution before reaching the eggs.

Since β -indole acetic acid may not enter the eggs readily in its highly dissociated state at pH 8.0 (although it was physiologically active in retarding development), its effect has also been tested at pH 6.0, again using the strongest concentration that did not considerably delay development. The permissible concentration at pH 6.0 was much less: 5 mg. per liter. In both experimental and control vessels the eggs were placed in acidified sea water at $2\frac{1}{2}$ hours after fertilization. They were irradiated with 2.8 units of λ 2537 Å at 8 hours after fertilization, and immediately after the irradiation the β -indole acetic acid was added to half of the vessels. The averaged results of 4 experiments, involving 742 control eggs and 670 experimental eggs to which β -indole acetic acid was added, show that 70.6 per cent of the control eggs formed rhizoids on the halves away from the radiation, and 69.1 per cent of the experimental eggs did so. The results of the individual experiments agree with this average result, and it may be concluded that β -indole acetic acid does not increase the sensitivity of the eggs at all at pH 6.0.

Other experiments were carried out in which the β -indole acetic acid was added some hours before irradiation. These experiments gave similar results, although the presence of even 5 mg. per liter of β -indole acetic acid at the time of irradiation slightly reduced the dosage of ultraviolet received by the experimental eggs, by absorption.

The Effect of Auxin on the Inhibiting Action of Strong Dosages of Ultraviolet

The preceding series of experiments tested the effect of β -indole acetic acid on the sensitivity of the eggs to relatively small unilateral doses of λ 2537 Å, as indicated by the positions at which the rhizoids formed. The object of this series of experiments is to find out whether the effects of heavy doses of λ 2537 Å, which are so strong as greatly to delay and inhibit rhizoid formation and cell division, are mitigated or relieved by

the addition of β -indole acetic acid. If the delay and inhibition of rhizoid formation is due primarily to the destruction of auxin in the egg cells, addition of β -indole acetic acid after irradiation should relieve the symptoms. Four experiments were carried out in which the eggs received a dosage of 20,000 units. The irradiation began 7.7 hours after fertilization and lasted approximately 38 minutes. Immediately after the irradiation, 150 mg. per liter β -indole acetic acid was added to the medium of half the vessels. The pH of all media was 8.1.

Normal non-irradiated eggs have all formed rhizoids at 24 hours after fertilization (Whitaker, 1936). The eggs irradiated with 20,000 units were retarded, however, so that less than half had formed rhizoids at 48 hours when counts were made in the present instance. The averaged results of the four experiments, involving 790 control eggs and 780 experimental eggs to which β -indole acetic acid was added, show that 35.2 per cent of the control eggs had formed rhizoids at 48 hours after fertilization, and 36 per cent of the experimental eggs had done so. The difference is entirely without significance and the conclusion is that β -indole acetic acid does not revive heavily irradiated eggs, or relieve their retardation or inhibition. This conclusion is further supported by the results of a few experiments in which 40,000 and 50,000 units of λ 2537 Å were applied. These heavy dosages greatly retarded and inhibited rhizoid formation, but the addition of β -indole acetic acid (150 mg. per liter at pH 8.0; 5 mg. per liter at pH 6.0) had no effect.

The Effect of pH on the Response to Ultraviolet

The effect of hydrogen ion concentration at the cell surface upon the response to ultraviolet is of interest because it provides further information about the reactions brought about in the protoplasm by the ultraviolet. Therefore experiments were designed to compare the sensitivity of the eggs to λ 2537 Å when the pH of the medium was 8.0 and 6.0. In each of 7 experiments two culture vessels contained eggs in medium at pH 8.0 and two contained eggs in medium at pH 6.0. Eggs were placed in medium at pH 6.0 2½ hours after fertilization, and all eggs were irradiated with 2.8 units at 8 hours after fertilization. In every one of the 7 experiments the eggs in medium at pH 6.0 responded more strongly. The overall average, based on 918 eggs at pH 8.0 and 1033 eggs at pH 6.0, shows that 65 per cent formed rhizoids on the halves away from the ultraviolet at pH 8.0 while 75 per cent did so at pH 6.0. This significant difference shows that the eggs are quite appreciably more sensitive to λ 2537 Å when they are in acidified medium. The eggs used in this and certain other series of experiments were less sensitive than those used to obtain the results shown in Fig. 1, but this is not regarded

as important since changes of this order are commonly found in different collections, especially at different times.

Another group of experiments was carried out to test the effect of pH of the medium on the sensitivity of the eggs to the retarding and inhibiting effects of strong dosages. In three experiments eggs were irradiated with 30,000 units of ultraviolet. The mid-point of the period of irradiation was at 8 hours after fertilization. In each experiment eggs in two vessels were placed in normal sea water at pH 8.0, while eggs in two other vessels were placed in sea water acidified to pH 6.0 at 2½ hours after fertilization and remained in this acidified medium thereafter. In all, 1049 eggs were irradiated and reared at pH 8.0, and 1358 eggs were irradiated and reared at pH 6.0. Non-irradiated control eggs have all formed rhizoids at 24 hours after fertilization (Whitaker, 1936), but the development of eggs irradiated with 30,000 units of λ 2537 Å is strongly retarded and inhibited. At 48 hours after fertilization, 6 per cent of the irradiated eggs at pH 8.0 had formed rhizoids, while 25 per cent of the eggs at pH 6.0 had formed rhizoids. Each experiment separately shows essentially the same relation. One of the experiments was carried further, until eggs that had not formed rhizoids began to cytolize. In this experiment, at 130 hours after fertilization, 32 per cent of the eggs at pH 8.0 had formed rhizoids while 61 per cent of those at pH 6.0 had done so. In another experiment approximately 400 eggs were irradiated with 50,000 units at pH 8.0, and a like number at pH 6.0. At 96 hours after fertilization none of the eggs at pH 8.0 had formed rhizoids, while 7 per cent of the eggs at pH 6.0 had formed rhizoids.

These results with strong dosages of λ 2537 Å show that the eggs are less sensitive to the damaging effects of strong ultraviolet when in medium at pH 6.0 than they are in medium at pH 8.0. Acidification of the medium thus has an opposite effect on sensitivity to strong damaging doses, compared with its effect on the sensitivity to polarity determination by moderate unilateral irradiation. This opposite effect of acidification suggests that different factors are involved in the two phenomena. Absorption measurements showed that normal sea water and acidified sea water transmit λ 2537 Å equally well, and it should also be noted that non-irradiated control eggs form rhizoids, and early rhizoids grow, at essentially the same rate in medium at pH 8.0 and in medium at pH 6.0.

DISCUSSION

The results presented in Fig. 1 show that the position of the rhizoid formation is extremely sensitive to ultraviolet light of the wave-length

2537 Å, and that the response of populations of eggs is proportional, over a wide range, to the logarithm of the dosage. This relation of response to dosage is similar to that already shown for λ 2804 Å (Whitaker, 1941), and similar to the dosage-response relation in the polarized plasmolysis of *Fucus* eggs after unilateral irradiation with λ 2804 Å (Reed and Whitaker, in press).

Auxin has been found to be present in *Fucus* eggs by du Buy and Olson (1937), and van Overbeek (1940) has shown that auxin occurring naturally in brown algae is very probably β -indole acetic acid. It was pointed out in the Introduction that destructive wave-lengths of ultraviolet might act on the *Fucus* egg primarily by inactivating or destroying auxin, and that, if so, addition of β -indole acetic acid might be expected to counteract or reduce the effect of the ultraviolet. The present experiments show, however, that β -indole acetic acid has very little effect on the sensitivity of the eggs to small doses of ultraviolet at pH 8.0, and none at all at pH 6.0. The slight effect found at pH 8.0 is not in the direction of reducing the effect of the ultraviolet. It may be due to a slight acidifying action of the β -indole acetic acid after entrance into the cell since acidity causes an increased sensitivity. β -indole acetic acid does not relieve or counteract the retarding and inhibiting effects on rhizoid formation and cell division of strong doses of ultraviolet at pH 8.0 or at pH 6.0. It thus appears that the ultraviolet does not act primarily by inactivating or destroying auxin, either in determining the developmental polarity or in inhibiting rhizoid formation.

Acidification of the medium to pH 6.0 appreciably increases the sensitivity of the eggs to the polarity-determining effects of moderate dosages of unilateral λ 2537 Å. On the other hand, acidification of the medium considerably decreases the sensitivity of the eggs to the retarding and inhibiting effects of strong dosages. This suggests that the two phenomena depend on different actions of the ultraviolet.

SUMMARY

1. When *Fucus* eggs are irradiated unilaterally with suitable moderate doses of λ 2537 Å, the rhizoids form on the non-irradiated halves of the eggs.

2. The response of populations of eggs is proportional, over a wide range, to the logarithm of the dosage.

3. When moderate doses of unilateral ultraviolet are followed by prolonged exposures to white light from the opposite sides of the eggs, the rhizoids form on the surfaces that were irradiated with ultraviolet. Therefore, the ultraviolet does not determine the position of rhizoid

formation merely by causing extensive, general damage and incapacitation of the surface upon which it falls.

4. β -indole acetic acid (auxin) has very little effect on the sensitivity of the eggs to moderate doses of ultraviolet at pH 8.0, and none at pH 6.0.

5. β -indole acetic acid does not revive eggs in which rhizoid formation and cell division have been retarded or inhibited by heavy doses of λ 2537 Å. Therefore the ultraviolet does not appear to act on the rhizoid formation primarily by affecting or destroying auxin.

6. The sensitivity of the eggs to the polarity-determining effects of moderate unilateral dosages of λ 2537 Å is considerably increased in sea water acidified from pH 8.0 to 6.0.

7. The sensitivity of the eggs to the retarding and inhibiting effects of strong dosages, on the other hand, is considerably decreased in medium acidified to pH 6.0. The two phenomena (directive effect of small doses and inhibitory effect of large doses) thus appear to depend on different actions of the ultraviolet.

The author is indebted to Mr. William E. Berg for assistance in carrying out the experiments.

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THE GAS CONTENT OF THE SWIMBLADDER OF THE ROCK BASS, *AMBLOPLITES RUPESTRIS*, IN RE- LATION TO HYDROSTATIC PRESSURE

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INTRODUCTION

A number of years ago Haldane suggested that the gases in the swimbladder of a physoclistomous fish are of blood origin; i.e., they are secreted into the swimbladder. Evidence has been secured in favor of such an hypothesis by Hall (1924), Powers (1932), Von Ledeboer (1937), and Black (1940). However, Krogh (1941) states, "This suggestion is very plausible, but even if proved would not go far to explain the gas secretion in this organ." The work of Powers, Jacobs, and Meesters and Nagel suggests the fundamental rôle of carbon dioxide in the physiology of the swimbladder. Perhaps it is necessary to know the limits of hydrostatic adjustment, the effect of barometric pressure changes on the general physiology of the fish, and the relation between the partial pressures of the gases in the swimbladder and the hydrostatic pressure before the physiology of the rete mirabile and the gas gland can be thoroughly understood. Many facts concerning the physiology of the swimbladder must be known before the rôle of this organ can be definitely established in the adjustment of fish to adverse conditions at certain levels, in the adjustment to barometric pressure changes, and the failure of adjustment in "sudden" mortality of fish during times of lake turnover.

The work presented below was performed with the hope of establishing a basis for further work toward the possible solution of these problems.

APPARATUS AND METHOD

An apparatus for the accurate control of hydrostatic pressure was designed, which consisted of a steel tank with a capacity of 32 gallons and capable of withstanding a pressure of 180 pounds. The tank was fitted with two 1½-inch, two 1-inch, and two half-inch tapings, a "sight glass" of vellemoid glass 2½ inches in diameter, and one hand-hole. The interior of the tank was lighted with a 40-watt bulb inclosed

in an explosion-proof globe purchased from the Crause-Hinds Company of Syracuse, New York. Pressure was maintained by a piston pump, the model "Marvel" constructed by the Deming Pump Company of Salem, Ohio. Under the experimental conditions the capacity of this pump was approximately two gallons per minute.

Pressure below one atmosphere was obtained by connecting the outlet of the tank to the pump, and the inlet to the lake. A by-pass was placed between the tank and the pump. Valves were placed between the tank and lake, tank and pump, and in the by-pass leading to the lake. The tank was filled completely with water, closed, and the pump started

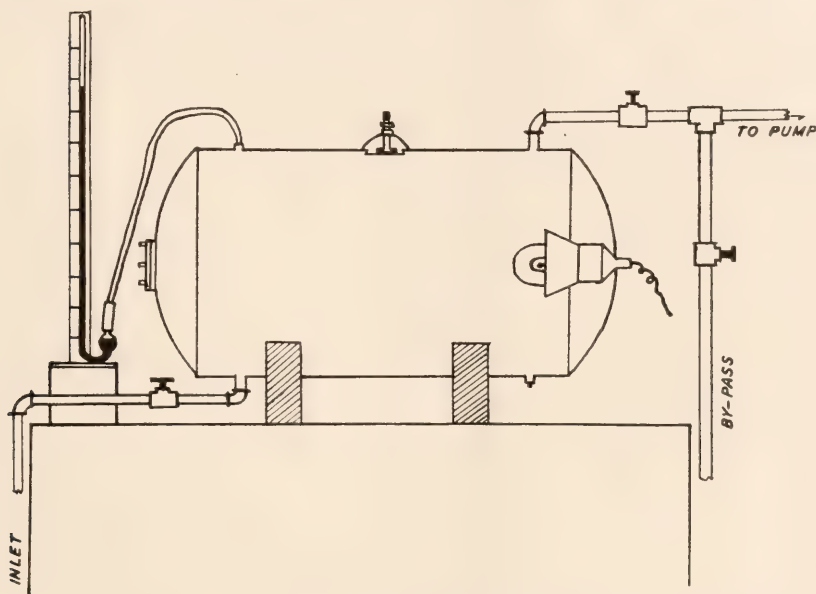


FIG. 1. Diagram of experimental apparatus, showing the arrangement used to produce pressure below one atmosphere.

with the by-pass open. When the pump had reached its capacity the by-pass was closed, and all the water was sucked from the lake through the tank. With this arrangement a pressure of 540 mm. Hg, equal to an altitude of 8,000 ft., was obtained. Figure 1 shows the arrangement of connections necessary to obtain this pressure.

The apparatus was thoroughly checked for air leaks by analyzing samples of water from the tank on the Van Slyke Blood Gas Analysis Apparatus and comparing with the analysis of samples taken from the open lake from which the water was pumped. In no case was there evidence that air was being forced into the water by the pump. The

gaseous content of the lake water remained practically constant throughout the experimental period (See Table I).

Hydrostatic pressure was measured by a gauge which was calibrated before the experiments were carried out and checked again at the end of the work. Low pressures were measured by means of a mercury manometer.

Gas was removed from the swimbladders of the experimental fish by an apparatus which consisted of two tonometers, a leveling bulb, and a hypodermic needle. The second tonometer was filled with mercury, as was the needle and its connection, and a partial vacuum was created in the first tonometer. The hypodermic needle was then inserted into the lumen of the swimbladder, the stopcocks opened, and gas was sucked into the second tonometer. All gas samples were removed while the

TABLE I

Analyses of the oxygen and nitrogen in the lake water compared with the amounts of these gases in the water of the tank at the time of the experiment.

No. Exp.	Average Pressure in Tank	O ₂ in Tank	O ₂ in Lake	N ₂ in Tank	N ₂ in Lake
	mm. Hg	vol. per cent	vol. per cent	vol. per cent	vol. per cent
6	761	.57	.52	1.13	1.11
12	2218	.77	.73	1.26	1.23
14	2229	.59	.71	1.11	1.18
19	1420	.67	.72	1.12	1.14
20	1147	.60	.60	1.33	1.33
24	1153	.72	.77	1.09	1.12
25	996	.91	.81	1.08	1.08
27	540	.79	.72	1.26	1.30
28	540	.79	.77	1.35	1.32

fish were submerged. The gas was analyzed by the use of the Henderson-Bailey Modification of the Haldane Apparatus.

The rockbass, *Ambloplites rupestris*, was selected as the experimental animal because it has a closed type of swimbladder, survives handling well, and recovers quickly from pressure effects. The blood of this fish is perhaps better buffered than such species as sheepshead and perch (Powers et al, 1939). The rockbass survives relatively high tensions of carbon dioxide.

The experimental procedure was as follows. Three rockbass, which had been removed from a shallow live-box where they had been confined for two days, were placed in the tank and the pressure was gradually increased to the point desired, where it was maintained until the fish had reached a state of hydrostatic equilibrium. This condition is defined as being that state in which the fish make little effort to maintain their

position; i.e., they swim on an even keel. When it was evident that this condition had been established (by observation through the "sight glass"), the fish were removed from the tank and gas samples were taken, usually within two minutes after the fall of pressure.

DISCUSSION OF EXPERIMENTAL DATA

Determinations of the swimbladder gas contents were made on fish which had been subjected to the following total pressures (hydrostatic plus atmospheric): 540 mm. Hg, 751 mm. Hg, 760 mm. Hg, 996 mm. Hg, 1148 mm. Hg, 1417 mm. Hg, and 2223 mm. Hg. The fish were removed from the tank when they had established a state of hydrostatic equilibrium. Several experiments were carried out at each pressure level, and as there were usually slight differences in pressure, the above pressures are averages. The data of the experiments are tabulated in Table II. The results obtained from the analyses of swimbladder gas taken from fish subjected to 750 and 751 mm. Hg are designated "controls." Those obtained at 760 mm. Hg were the results of analyses of swimbladder gas taken from fish which were removed from a live-box where they had been kept for at least two days. The results at 750 mm. Hg were obtained from fish which had been placed in the tank for six hours with no pressure registering, but with a constant flow of water passing through the tank. The average percentage of carbon dioxide in the swimbladders of fish taken from the live-box was 2.02 per cent, and 1.54 per cent in the case of the fish placed in the tank for six hours. The oxygen percentage in the "control" experiment at 750 mm. Hg was 14.98 per cent, while the percentage of oxygen in the swimbladder of fish taken from the live-box was 15.93 per cent, on the average. The nitrogen in the "control" was 83.63 per cent, and in the fish taken from the live-box the percentage of nitrogen was 81.92 per cent.

At the suggestion of Dr. Alfred C. Redfield, a constant volume of the swimbladder was assumed for fish in a state of hydrostatic equilibrium at any hydrostatic pressure. For example, fish subjected to 750 mm. Hg pressure, the "control," were assumed to have a certain volume of the swimbladder, and fish which were in a state of hydrostatic equilibrium at 996 mm. Hg would have the same volume which they had at 750 mm. Hg, since the density of the fish is equal to the density of the water, and the mass of the fish is unchanged when the fish have reached a state of hydrostatic equilibrium. This assumption gives a basis for calculating the volume of each gas, the gain or loss of each gas in the swimbladder in making the equilibration from the level of the "control," and the rate of gas deposition at each pressure level. In Table III the milliliters of each gas in 100 ml. of swimbladder gas at a given hydrostatic pressure

is corrected to normal conditions of temperature and pressure, that of the "control."

The results at the various pressure levels will be discussed with reference to the volume of each gas at normal temperature and pressure, beginning at the reduced pressure of 540 mm. Hg. When fish had

TABLE II

Results of the analyses of swimbladder gas obtained from the rockbass, *Ambloplites rupestris*, which had attained a state of hydrostatic equilibrium at the total pressures given in column No. 2.

No. Exp.	Total Pressure	Time	Temp. of Water	CO ₂	O ₂	N ₂
	<i>mm. Hg</i>	<i>hours</i>	<i>° C.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
26	540	24	26.4	0.85	3.94	95.21
27	540	24	26.4	0.73	6.37	92.93
28	540	22.5	25.5	0.75	4.22	95.02
28	540	22.5	25.5	0.72	4.78	94.49
28	540	22.5	25.5	0.72	4.19	95.08
av.	540	23	26	0.77	4.50	94.54
1	750	6.0	11.6	1.23	14.79	83.98
1	750	6.0	11.6	1.54	13.50	84.96
1	750	6.0	11.6	1.79	16.20	82.21
2	751	6.0	13.9	.97	10.01	89.02
2	751	6.0	13.9	1.91	17.98	80.11
2	751	6.0	13.9	1.67	16.30	83.03
3	751	6.0	15.5	1.81	18.94	79.25
3	751	6.0	15.5	1.42	13.10	86.48
av.	750	6.0	13.6	1.54	14.98	83.63
32	759	1.b.*	21.6	1.15	18.17	80.68
32	759	1.b.	21.6	1.87	20.87	77.26
6	761	1.b.	22.2	3.37	13.68	82.44
6	761	1.b.	22.2	1.67	11.02	82.31
av.	760		22.0	2.02	15.93	81.92
25	996	10.5	26.6	6.73	37.11	56.12
25	996	10.5	26.6	3.02	35.08	61.89
25	996	10.5	26.6	4.66	25.86	69.46
av.	996	10.5	26.6	4.73	32.68	61.26

* 1.b., live box.

reached hydrostatic equilibrium at 540 mm. Hg, it was found that all gases had been partially removed from the swimbladder. At this pressure the amount of carbon dioxide in 100 ml. of swimbladder gas was only 0.7 ml., and the amount of oxygen on the same basis was only 4.5 ml., while the nitrogen in 100 ml. of swimbladder gas was 94.5 ml. When corrected to normal conditions of temperature and pressure, the

above volumes become 0.5 ml. CO_2 , 3.1 ml. O_2 , and 65 ml. N_2 . The amounts of each gas in 100 ml. of swimbladder gas removed from fish in the "control" at 750 mm. Hg and 13.6°C . are 1.5 ml. CO_2 , 15.0 ml. oxygen, and 83.6 ml. nitrogen. Thus it can be seen that one milliliter of carbon dioxide per 100 ml. of swimbladder gas has been lost along with 11.9 ml. of oxygen and 17.6 ml. of nitrogen. (Table III gives the milliliters of each gas gained or lost per 100 ml. of swimbladder gas in

TABLE II—*Continued*

No. Exp.	Total Pressure	Time	Temp. of Water	CO_2	O_2	N_2
	<i>mm. Hg</i>	<i>hours</i>	$^\circ \text{C}$.	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
20	1147	24.1	25.5	16.45	35.67	47.88
21	1145	23.7	26.1	3.50	45.28	51.20
21	1145	23.7	26.1	8.29	44.50	47.20
21	1145	23.7	26.1	9.36	34.38	55.81
24	1153	25.1	25.5	15.24	35.22	49.45
24	1153	25.1	25.5	11.88	36.92	51.13
25	1153	25.1	25.5	4.46	39.60	55.94
av	1147	24.4	25.5	9.88	38.86	51.24
19	1420	38.5	26.1	6.69	51.60	41.71
19	1420	38.5	26.1	13.24	46.89	39.79
19	1420	38.5	26.1	11.80	32.17	55.83
5	1414	47.5	22.2	15.27	42.24	42.23
5	1414	47.5	22.2	11.27	40.89	46.84
5	1414	47.5	22.2	2.35	56.85	40.80
av.	1417	43.0	24	10.17	45.10	44.53
12	2218	56.5	21.6	8.43	55.57	36.00
12	2218	56.5	21.6	7.40	61.30	31.31
12	2218	56.5	21.6	8.53	60.26	31.14
13	2223	56.5	21.6	7.97	62.17	29.92
13	2223	56.5	21.6	10.99	42.07	46.94
14	2229	66.5	25	9.02	52.05	35.35
14	2229	66.5	25	9.75	45.90	43.35
14	2229	66.5	25	8.39	61.18	40.48
av.	2223	61.0	22.6	8.96	54.73	36.31

equilibrating to the various levels of hydrostatic pressure.) In order to reach equilibrium at 540 mm. Hg, approximately 27 per cent of the gas in the swimbladder at equilibrium at 750 mm. Hg would have to be removed. Since only 5 per cent of the gas at 750 mm. Hg is carbon dioxide and oxygen, equilibrium at 540 mm. Hg cannot take place unless nitrogen is removed. Unless nitrogen is removed, the rockbass could not come to equilibrium at a pressure of less than 635 mm. Hg. Brown (1939)

offers experiments on the guppy as evidence of nitrogen deposition at reduced pressures. The results on the rockbass do not show nitrogen deposition in the adjustment to any pressure, as will be pointed out below. However, Brown found that the guppy did not make adjustment to pressure reductions of more than 125 mm. Hg. If the swimbladder, as stated above, did not contain more than 16 per cent carbon dioxide plus

TABLE III

The milliliters of each gas gained or lost per 100 ml. of swimbladder gas were calculated from the data secured at each pressure level as given in Table II, assuming a constant volume of the swimbladder at hydrostatic equilibrium.

Average Total Pressure	CO ₂	O ₂	N ₂	
<i>mm. Hg</i>	<i>ml</i>	<i>ml</i>	<i>ml</i>	
540	0.7	4.5	94.5	per 100 ml.
	0.5	3.1	65.0	corrected NTP
	-1.0	-11.9	-17.6	gain or loss
750	1.5	15.0	83.6	"Control"
				NTP = 750 mm. Hg, 13.6° C.
760	2.0	15.9	81.9	per 100 ml.
	1.9	15.6	86.4	corrected NTP
	+0.4	+0.6	-3.2	gain or loss
996	4.7	32.7	61.3	per 100 ml.
	6.0	41.5	78.8	corrected NTP
	+4.5	+26.5	-4.8	gain or loss
1147	9.9	38.9	51.2	per 100 ml.
	14.5	57.3	75.3	corrected NTP
	+13.0	+42.3	-8.3	gain or loss
1417	10.2	45.1	44.5	per 100 ml.
	18.3	81.9	80.6	corrected NTP
	+16.8	+66.9	-3.0	gain or loss
2223	8.9	54.7	36.3	per 100 ml.
	25.6	156.5	103.8	corrected NTP
	+24.1	+141.5	+20.2	gain or loss

oxygen, adjustment could not be made to pressures below 635 mm. Hg without the removal of nitrogen.

In the adjustment to 996 mm. Hg it was found that 4.5 ml. carbon dioxide and 26.5 ml. oxygen were deposited in the swimbladder while 4.8 ml. nitrogen were removed for every 100 ml. of swimbladder gas.

At hydrostatic equilibrium at the total pressure of 1147 mm. Hg there were deposited in the swimbladder 13 ml. carbon dioxide and 42.3 ml. oxygen; and 8.3 ml. nitrogen were removed per 100 ml. of swimbladder gas.

The adjustment to the hydrostatic pressure of 1417 mm. Hg was the result of the deposition of 16.8 ml. carbon dioxide and 66.9 ml. oxygen per 100 ml. of swimbladder gas, while 3 ml. of nitrogen were removed.

Likewise there was a deposition of 24.1 ml. carbon dioxide and 141.5 ml. oxygen per 100 ml. of swimbladder gas in the adjustment to 2223 mm. Hg pressure. In this case nitrogen appeared to have increased. The slight deviation of the nitrogen in several experiments from the level of the "control" is believed to be due to an error in the use of average figures, originally caused by the failure of some fish in the experiment to have reached complete equilibrium.

Figure 2 is a graphic representation of the data compiled in Table III in which the milliliters of the three gases per 100 ml. of swimbladder gas are plotted against the hydrostatic pressure in mm. Hg. It can be seen that the nitrogen fluctuates only slightly. It will be noted that the volume of carbon dioxide plotted against the hydrostatic pressure forms a curve which tends to level off as the pressure increases. The oxygen forms a nearly straight line, which indicates that the deposition of this gas is largely responsible for the adjustment to hydrostatic pressure. At the total pressure of 2223 mm. Hg, over half of the pressure exerted against the swimbladder wall is due to oxygen. Carbon dioxide partial pressure (200 mm. Hg) is equal to only one-tenth of the total pressure. Meesters and Nagel (1934-35) state that 86 per cent of the "Ersatzgas" is composed of carbon dioxide. Their results were obtained for only the first hour of gas deposition. If the gas entering the swimbladder is composed of 86 per cent carbon dioxide for the duration of gas deposition, carbon dioxide partial pressure would be much higher than 200 mm. Hg. In as much as the pressure of oxygen at 2223 mm. Hg is half the total pressure, it is impossible that the secretion of carbon dioxide continued to form 86 per cent of the total secretion and it is quite likely that the ratio of carbon dioxide to oxygen secreted changes greatly during equilibration. The ratio of these two gases varies considerably at equilibrium. In Table IV the milliliters of carbon dioxide and oxygen are the corrected volumes of these gases in 100 ml. of swimbladder gas observed at equilibrium at the various pressure levels.

The average ratio is 6.5; i.e., there are 6.5 times more oxygen than

carbon dioxide in the swimbladder at hydrostatic equilibrium. Meesters and Nagel found 6.1 times more carbon dioxide than oxygen in the gas which entered the swimbladder during the first hour of gas deposition.

Two series of experiments were performed which cast some light on the rate of gas deposition, the rôle of carbon dioxide after the first two hours of gas deposition, and upon the relation of the amount of carbon

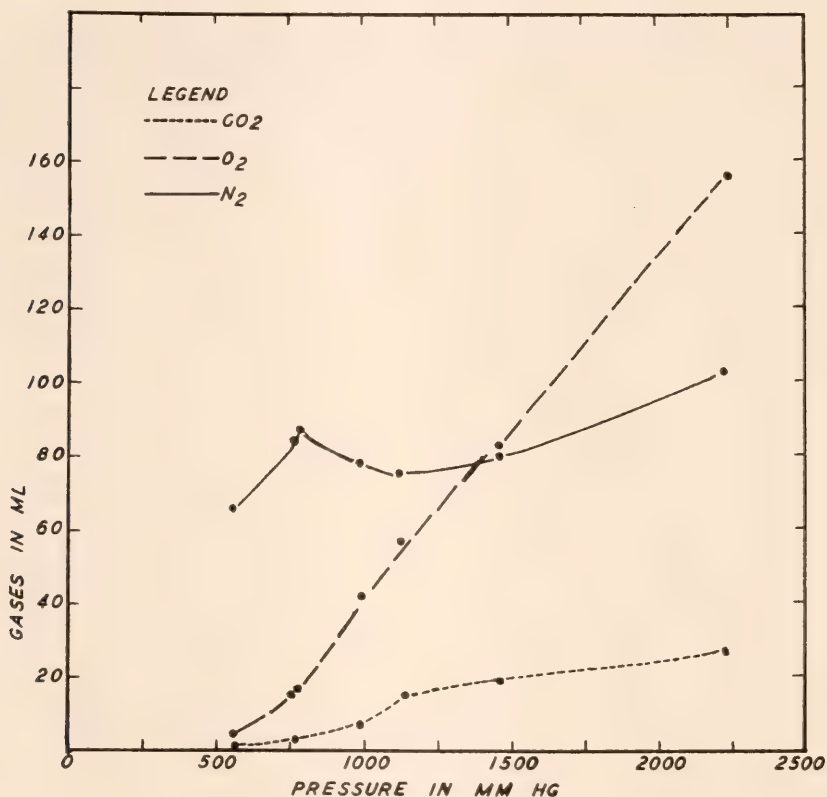


FIG. 2. A graphic representation of the data of Table III wherein the volume of gas is calculated on the assumed volume of 100 ml. for the swimbladder at hydrostatic equilibrium.

dioxide deposited to the strength of the stimulus—the hydrostatic pressure. In one series of experiments the pressure, 996 mm. Hg, remained constant, and the fish were placed under this pressure for various lengths of time. The results of analyses of swimbladder gas taken from fish which had been subjected to 996 mm. Hg for two hours, 10.5 hours, and

TABLE IV

Hydrostatic Pressure	CO ₂	O ₂	Ratio O ₂ /CO ₂
<i>mm. Hg</i>	<i>ml.</i>	<i>ml.</i>	
540	0.5	3.1	6.2
750	1.5	15.0	10.0
760	1.9	15.6	8.2
996	6.0	41.5	6.9
1147	14.5	57.3	3.9
1417	18.3	81.9	4.4
2223	25.6	156.5	6.1

24.6 hours are given in Table V. Figure 3 is a graphic representation of these data wherein the partial pressures of the various gases are plotted against "time." It can be seen that the partial pressure of carbon dioxide is rapidly increased, from the point of the "control" during the first two hours. The "control" is the average amount of carbon dioxide in the swimbladder of fish at the surface of the lake. The rate of increase of carbon dioxide falls off during the next eight hours. When the fish are allowed to remain at this pressure (996 mm. Hg) for 24.6 hours, which is 12.5 hours after they have reached a state of hydrostatic equilibrium, carbon dioxide partial pressure has decreased by

TABLE V

These data were obtained from analyses of swimbladder gas taken from fish which had been subjected to 996 mm. Hg hydrostatic pressure for the durations listed in column No. 2.

No. Exp.	Time	Total Pressure	CO ₂ Content	CO ₂ Pressure	O ₂ Content	O ₂ Pressure	N ₂ Content	N ₂ Pressure	Temp. of Water
	<i>hours</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>°C.</i>
29	2.0	993	7.65	74.2	25.70	248.3	66.66	644.0	26.8
29	2.0	993	3.42	33.0	17.30	167.1	79.26	765.7	26.8
29	2.0	993	1.01	9.7	26.60	256.9	72.39	699.3	26.8
av.			4.03	39.0	23.20	204.5	72.81	703.0	
25	10.5	996	6.73	64.6	37.11	353.6	56.12	559.3	25.8
25	10.5	996	3.02	29.0	35.08	337.1	61.89	594.7	25.8
25	10.5	996	4.66	44.7	25.86	248.5	69.46	677.5	25.8
av.			4.73	46.1	32.68	325.1	61.26	610.5	
23	24.6	999	2.13	20.8	31.27	305.9	66.60	651.5	22.7
23	24.6	999	3.81	37.2	35.22	344.5	60.96	596.4	22.7
23	24.6	999	2.06	20.1	45.45	444.5	52.46	513.2	22.7
av.			2.67	26.0	37.33	365.0	60.00	587.0	

two-fifths of its value at 10.5 hours. Concomitantly with the rise in carbon dioxide, there is an 8 per cent increase in oxygen during the first two hours of stimulation. During the next eight hours the oxygen increased 12 per cent, while only 2 per cent increase was shown for this gas during the last 14 hours. It has been suggested by Dr. Laurence Irving¹ that the delay in the maximum of oxygen in contrast to carbon dioxide in the swimbladder may be due to the relatively large volume of

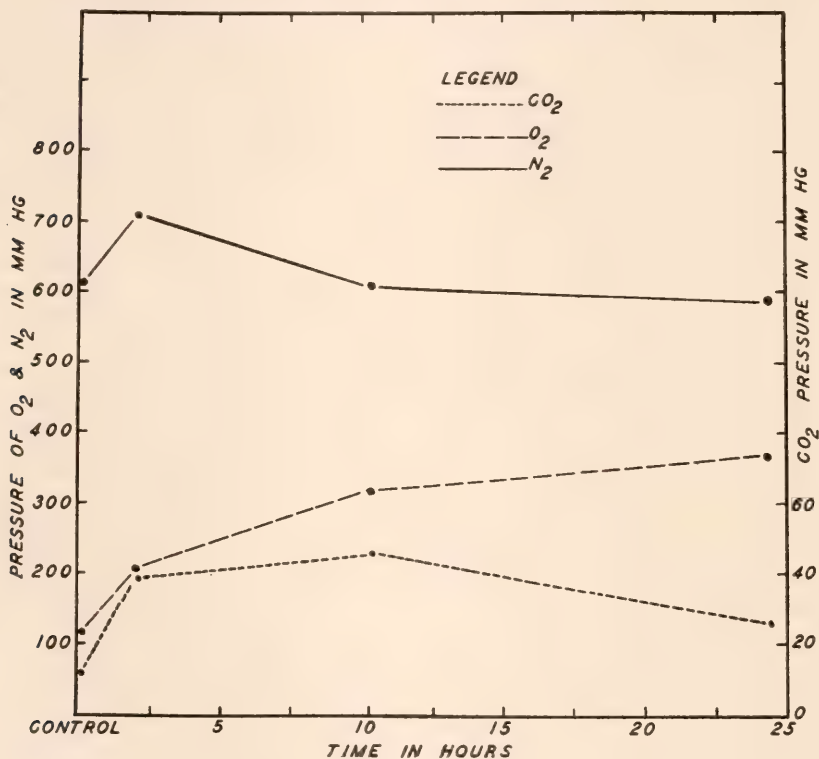


FIG. 3. A graphic representation of the data given in Table V.

oxygen which must be moved. The partial pressure of the nitrogen falls to the level of the atmospheric nitrogen by the end of the 24-hour period. Thus it can be seen that nitrogen is not deposited. Oxygen and carbon dioxide are rapidly deposited during the first two hours, and unless carbon dioxide is being removed from the swimbladder as it is being deposited, oxygen is entering the swimbladder as rapidly as

¹ Dr. Laurence Irving by personal communication.

carbon dioxide, if not more so, during the first two hours. It hardly seems likely that carbon dioxide is being removed from the swimbladder during the early part of adjustment, for, in the light of Meesters' and Nagel's and Jacob's work, carbon dioxide deposition may account for small adjustments to hydrostatic pressure. Carbon dioxide could not diffuse out of the bladder rapidly during this length of time, since it has been proved by Meesters and Nagel (1934-35) that the "oval" is tightly closed during the early part of gas deposition. Fish subjected to a total pressure of 996 mm. Hg reach hydrostatic equilibrium in 10 hours. After this time carbon dioxide partial pressure decreased toward the level of the carbon dioxide partial pressure of the control. It appears that carbon dioxide is being removed from the swimbladder after the fish have attained a state in which they are capable of moving about without too much difficulty.

In another series of experiments fish were subjected to 1417 mm. Hg for various periods of time, from 2 hours to 73.5 hours. The results of the analyses of swimbladder gas are given in Table VI, and graphically illustrated in Fig. 4, in which the partial pressures of the gases are plotted against "time" in hours. The carbon dioxide increased sharply during the first two hours from 1.54 per cent of the control to 7.98 per cent at the end of the two-hour period. During the next five hours the carbon dioxide increased to 13.93 per cent, which is the highest partial pressure recorded for this gas at the total pressure of 1417 mm. Hg. From this time, 7.5 hours, to the longest period of adjustment, carbon dioxide percentage decreased to the lowest point, 4.22 per cent, in 73 hours. This percentage is equal to a partial pressure of 59.1 mm. Hg, which is still higher than the partial pressure of this gas in the control. This indicates that, even in 73 hours, fish which have been subjected to 1417 mm. Hg of pressure have not reached a state of gaseous equilibrium within the swimbladder. It is evident that after 38 hours carbon dioxide is being removed faster than it is entering the swimbladder. The highest average partial pressure for carbon dioxide in the swimbladder of fish subjected to 1417 mm. Hg is 192 mm. Hg, and the partial pressure of CO_2 in the swimbladder of fish subjected to 2223 mm. Hg of total pressure is 197 mm. Hg. Since the carbon dioxide curve levels off at 200 mm. Hg, it might be possible that this is the limit of the amount of carbon dioxide this particular species can deposit. In other words, the level of the carbon dioxide partial pressure may be a limiting factor to the hydrostatic adjustment. The highest average partial pressure for carbon dioxide in the swimbladders of fish subjected to 996 mm. Hg is 46.1 mm. Hg. Thus it can be seen that there

TABLE VI

These data were obtained by the analyses of swimbladder gas taken from fish which were subjected to 1417 mm. Hg hydrostatic pressure for the duration listed in column No. 2.

No. Exp.	Time	Total Pressure	CO ₂ Content	CO ₂ Pressure	O ₂ Content	O ₂ Pressure	N ₂ Content	N ₂ Pressure	Temp. of Water
	<i>hours</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>per cent</i>	<i>mm. Hg</i>	<i>°C.</i>
22	2.0	1415	8.82	122.6	36.92	513.2	54.26	754.2	26.1
22	2.0	1415	8.10	112.6	35.49	493.3	56.41	784.1	26.1
22	2.0	1415	6.04	83.9	19.20	266.9	74.16	1031.0	26.1
av.			7.98	106.3	30.53	424.4	61.61	856.4	
18	7.5	1415	13.72	190.3	30.05	416.8	56.10	778.1	28
18	7.5	1415	8.72	120.9	38.02	527.3	53.26	738.7	28
18	7.5	1415	19.17	265.9	26.87	372.7	53.93	748.0	28
av.			13.93	192.3	31.96	438.9	54.39	754.9	
17	13.7	1415	13.75	191.3	25.07	348.7	60.98	848.2	25.6
17	13.7	1415	11.06	153.8	23.67	329.3	65.27	908.3	25.6
17	13.7	1415	13.12	182.5	32.43	451.1	54.21	754.1	25.6
av.			12.67	175.8	27.09	376.3	61.14	836.9	
4	27.0	1416	13.70	196.3	30.86	430.8	55.44	773.9	22.2
4	27.0	1416	13.10	182.9	30.38	424.1	56.52	789.0	22.2
4	27.0	1416	11.10	154.5	38.44	536.6	50.86	710.0	22.2
av.			12.60	176.2	33.23	463.8	54.26	757.6	
19	38.5	1420	6.69	93.3	51.60	719.8	41.71	581.9	26.1
19	38.5	1420	13.24	184.7	46.89	655.8	39.79	565.1	26.1
19	38.5	1420	11.80	164.6	32.17	448.8	55.83	778.8	26.1
av.			10.38	147.5	43.55	606.1	45.77	641.9	
5	47.5	1414	15.27	212.8	42.24	588.8	42.23	588.7	22.2
5	47.5	1414	12.27	171.0	40.89	570.0	46.84	652.9	22.2
5	47.5	1414	2.35	32.7	56.85	792.5	40.80	576.6	22.2
av.			9.96	138.8	46.66	650.4	43.30	606.7	
10	66.0	1426	4.83	67.9	57.71	811.7	37.46	527.0	21.6
10	66.0	1226	10.06	141.4	48.90	688.0	41.04	577.3	21.6
av.			7.45	104.7	53.20	749.8	39.35	552.1	
30	73.3	1425	2.68	37.6	54.51	764.6	42.81	600.5	23.9
30	73.3	1425	8.42	118.1	42.72	598.3	48.70	683.3	23.9
30	73.3	1425	1.56	21.8	51.79	726.6	46.67	654.8	23.9
av.			4.22	59.1	49.73	696.5	46.06	646.2	

is a relationship between the partial pressure of carbon dioxide and the stimulus, the hydrostatic pressure. In the experiments carried out at 1417 mm. Hg the oxygen increased sharply during the first two hours with the initial increase in carbon dioxide, but, as in the experiments at

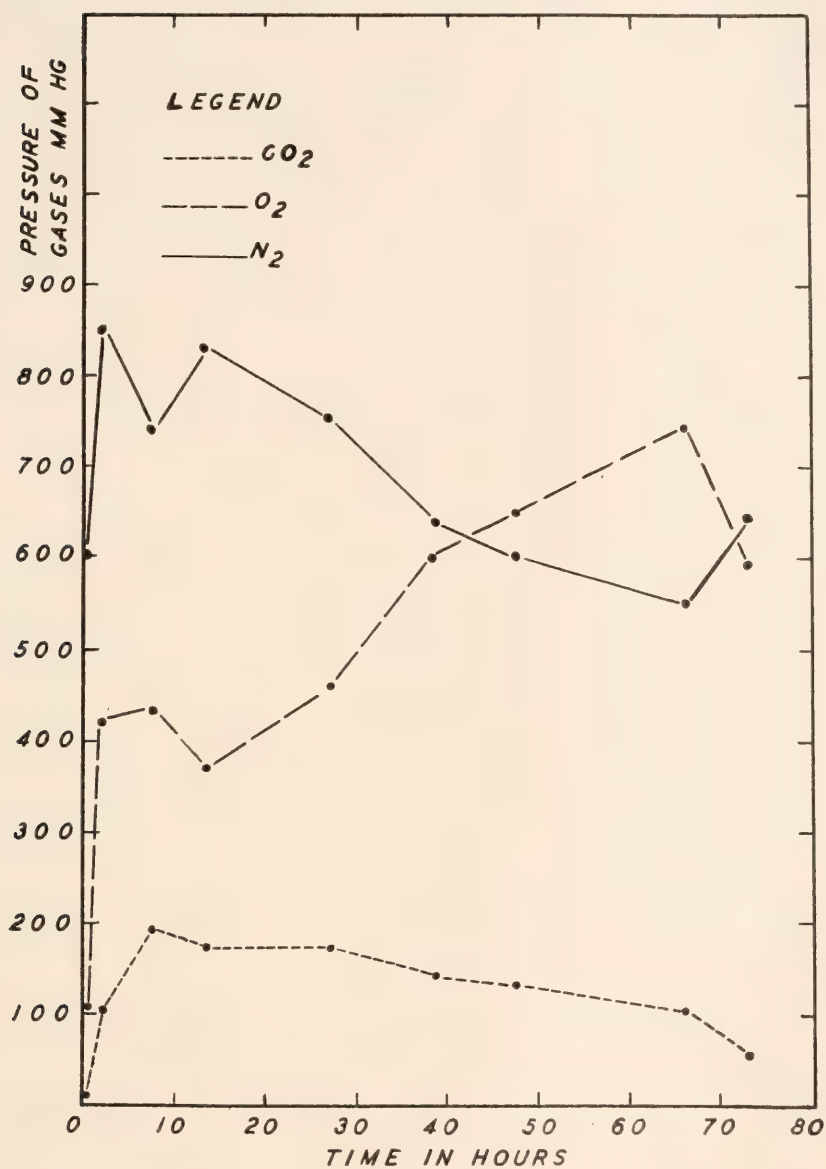


FIG. 4. A graphic representation of Table VI. The point 0 represents status of swimbladder gas of the control.

996 mm. Hg, the oxygen continued to increase after the carbon dioxide is decreasing. The nitrogen partial pressure decreases toward the level of the atmospheric nitrogen.

From these two series of experiments it is evident that both carbon dioxide and oxygen are increased sharply during the first two hours. After the fish have made a temporary adjustment to hydrostatic pressure, carbon dioxide is removed from the swimbladder faster than it is being deposited. The final adjustment results in a high partial pressure of oxygen, a carbon dioxide partial pressure near the level of the control, and a nitrogen pressure approximately equal to the atmospheric nitrogen partial pressure.

SUMMARY

1. Rockbass were subjected to various hydrostatic pressures ranging from 540 to 2223 mm. Hg and were observed to reach a state of equilibrium at each experimental pressure.

2. Oxygen deposition accounted for half of the adjustment at the higher hydrostatic pressures.

3. After hydrostatic equilibrium had been reached carbon dioxide diffused out of the swimbladder, and the carbon dioxide percentage decreased toward the level of this gas usually found in fish at the surface of the lake. This loss in volume by the outward diffusion of carbon dioxide was compensated for by the continued deposition of oxygen.

4. Nitrogen was not deposited, and appeared to be only passively important in the adjustment to the changes in hydrostatic pressure.

5. The final adjustment to hydrostatic pressure resulted in a high partial pressure of oxygen, a carbon dioxide partial pressure near the level usually found for this gas in fish at the surface, and a nitrogen partial pressure approximately equal to the partial pressure of this gas in the atmosphere.

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BUDS INDUCED IN CLYMENELLA TORQUATA BY IM-
PLANTS OF NERVE CORD AND NEIGHBORING
TISSUES DERIVED FROM THE MID-BODY
REGION OF WORMS OF THE
SAME SPECIES

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In an endeavor to learn more concerning the factors involved in the determination of the type of structure which will develop when a part of the body is lost, a variety of transplantation experiments have been carried out on the polychaete *Clymenella torquata*. It has been shown that when pieces of nerve cord with neighboring tissues from non-regenerating donors are inserted in the body wall of intact hosts, buds are frequently induced. An implant derived from the anterior end (segments 1-7) of a donor may give rise to a bud of either head or tail type (Sayles, 1940a). Whether a head or a tail forms depends to a large extent upon the segment of the host into which the implant is inserted. In the region between segments 1 and 9, head buds have been induced by anterior implants in about one case in six, but no tail buds have ever appeared. When inserted posterior to segment 12, this type of implant does not produce head buds but often results in the formation of an incomplete tail bud with the ventral part of the anal segment missing. The region of segments 10-12 is a transitional zone in which several types of well-differentiated buds may appear, including: (1) heads; (2) tails with the ventral parts of the anal segments missing as at posterior levels; (3) buds each with the ventral half of the anal segment missing and with a cone of new material projecting posteriorly into the open region; (4) buds each with a terminal region consisting of the dorsal half of an anal segment on the open, ventral side of which is a proboscis or similar part.

Implants taken from the posterior region of donors, on the other hand, will not produce head buds, regardless of the level of the host's body at which they are inserted. At posterior levels they produce complete tails. In the transitional zone they give rise to two definite types: (1) complete tails and (2) buds terminating in anal segments of which

only the ventral parts are present. At anterior levels these implants also frequently produce buds terminating in ventral parts of anal segments. In some cases the latter bear extra, conical parts dorsally.

Both anterior and posterior implants exhibit a definite polarity. Definitive head or tail buds are ordinarily not induced by anterior implants when they are inserted with their posterior ends exposed at the surface of the host's body. Posterior pieces, however, must be oriented with their posterior ends out if they are to result in differentiated buds.

The present paper, reporting on results obtained with implants derived from the mid-body region, is intended to complete the description for transplantation experiments in which both donors and hosts are non-regenerating worms. There are reported 277 cases in which im-

TABLE I

Results of implantation of mid-body pieces with anterior end exposed.

Level of insertion Source of implant, segments	Anteriorly			In mid-body			Posteriorly			Totals
	8-9	9-10	10-11	8-9	9-10	10-11	8-9	9-10	10-11	
Head buds	2	2		4	3	1				12
Double Tail			1							1
Tails, open ventrally...				2	1		2	3	1	9
Tails, complete							5	1		6
Indeterminate buds....	7	4	3	10	6	7			4	41
No new material	7	6	7	2	3	2	1	4	6	38
Died within 10 days...	2	3		4	3	1	1	4	3	21
Implant lost by host...	1	2	1	1				1	1	7
Totals	19	17	12	23	16	11	9	13	15	135

plants taken from this mid-body region, segments 8-11, were inserted in the three different regions of the body of *Clymenella*. As in previous experiments, the implants were inserted in the dorso-lateral parts of the segments. This work was carried on at the Marine Biological Laboratory, Woods Hole, during the summers of 1935-1940, inclusive. In the following account most of the buds are described as they were at the time of preservation or discard or, in some cases, a brief time before they were lost.

RESULTS

The results of 135 cases in which the anterior ends of the implants were exposed are summarized in Table I. The double tail bud consisted of a basal, rounded region bearing two small, partial, anal segments open

ventro-laterally, with the open sides turned slightly toward one another. Each of the buds designated "tail, open ventrally" terminated in a partial anal segment with the ventral half missing. In most of the buds of this type formed at posterior levels the anal-segment regions were large with only a very small mid-ventral region missing in each case. Buds described as "tail, complete" were so called because the anal segments were complete. The indeterminate buds included all of those not possessing at least a clear-cut portion of a head or tail segment. Some of these were elongate with more or less evidence of segmentation but with rounded terminal regions. Others were somewhat conical masses of new material, with occasionally one or more notopodial or neuropodial elements present.

TABLE II

Results of implantation of mid-body pieces with posterior end exposed.

Level of insertion	Anteriorly			In mid-body			Posteriorly		Totals
Source of implant. segments	8-9	9-10	10-11	8-9	9-10	10-11	9-10	10-11	
Head buds.....		1	2			2			5
Head-tail buds.....			1			1			2
Tail-cone bud.....			1						1
Tail, open dorsally.....			1		1	5			7
Tail, complete.....						4	3	10	17
Indeterminate buds.....	5	3	7	9	5	16	6	3	54
No new material.....	8	7	3	2	3	7	3	1	34
Died within 10 days.....	2	3	2	3	2	5	2	1	20
Implant lost by host.....	1			1					2
Totals.....	16	14	17	15	11	40	14	15	142

In the remaining 142 operations the posterior ends of the implants were at the surface of the host's body. These are summarized in Table II. Each of the head-tail buds terminated in a weakly differentiated peristomial region and had a partial anal segment, open dorsally, attached in the ventral region of the main bud a short distance from the peristomium. The tail-cone bud was unsegmented and terminated in a large, partial anal segment, the dorsal side of which was missing. On the dorso-lateral side of this anal segment, near its base, there was a small, conical mass of material.

From these data it is clear that implants derived from the level of segments 8-9 possess a distinct polarity. Although 15 definitive head or tail buds resulted from 51 operations in which the anterior ends of these implants were out, none of the 31 cases in which the posterior ends

of the implants were at the surface produced a head or tail bud. It is also interesting that implants from 8-9 produced no tails when inserted at anterior levels, no heads at posterior levels and both types in the transitional region. Both of the mid-body tails were formed at the tenth segment. In all of these respects these implants from segments 8-9 follow rather closely the behavior of implants from anterior sources.

Implants from segments 9-10 of donors, when oriented with their anterior ends out, produced results which resembled those of pieces made up of segments 8-9. The only marked difference was in the distinctly smaller number of tail buds produced by segments 9-10 at posterior levels of hosts. But these implants when inserted with their posterior ends out produced several well-differentiated buds. One was a fairly well formed head which developed at an anterior level, segment 7. One, formed at segment 10, was a small tail bud with only the ventral part of the anal-segment portion present. The other three, produced at segments 13 and 14, were small tail buds with complete anal collars. In distinct contrast with these results are those obtained with anterior pieces inserted with their posterior ends out, an arrangement in which definitive head or tail buds are very rarely produced.

Implants from segments 10-11 showed further transition toward the behavior typical of posterior pieces. Definitive head or tail buds appeared in 3 of 38 cases (about 8 per cent) in which the anterior ends of the implants were exposed. These were of three different types: a double tail, a fairly well-differentiated head, and a bud with only the dorsal part of the anal segment present. On the other hand, 27 out of 72 cases (about 37 per cent) in which the posterior ends of such implants were on the surface gave rise to head or tail buds. At anterior and mid-body levels these belonged to several different types. At posterior levels, however, only tails with complete anal segments were formed. Also the number of differentiated buds produced was much greater in proportion at posterior levels (67 per cent) than in the anterior or transitional regions.

DISCUSSION

In general these results with implants from mid-body sources are in accord with what one might expect from a study of buds formed from implants from the anterior and posterior regions of donors. In each case where a tail bud with partial anal segment arose from an implant inserted with its anterior end out, the dorsal part of the anal segment was the portion present regardless of the source of the implant. Evidence has been presented (Sayles, 1940*b*) that the ventral part of the

terminal segment is usually determined by the implant and the dorsal part usually by the host. It is clear, then, that although a partial tail bud may be induced at the anterior end of an implant, the nature of this structure is probably determined by the host. In the case of a partial tail bud produced by an implant with its posterior end out, it was always the dorsal part of the anal segment which was missing. These implants from the mid-body region showed further that the type of bud produced is determined through the interaction of implant and host.

When inserted anterior to the tenth segment, anterior implants produced heads but never tails (Sayles, 1940*a*). Posterior implants inserted in this anterior region produced no heads but frequently tail buds, all of whose anal segments lacked dorsal portions (Sayles, 1940*b*). Mid-body implants made up of the nerve cord of segments 8-9 or 9-10 gave results similar to those following the implanting of anterior pieces here. Pieces of nerve cord made up of segments 10-11, however, showed intermediate behavior. They produced both tails and heads, with the latter being formed even when the posterior ends of implants were exposed. This appearance of heads would not have been predicted from the study of implants from anterior and posterior sources.

In the mid-body region anterior pieces produced both head and tail buds with the latter frequently bearing extra parts which in some cases showed definite head features. Posterior pieces, however, were virtually limited to the formation of tail buds without extra parts. Pieces of nerve cord made up of segments 8-9 or 9-10 gave rise to both head and tail buds, with the latter not bearing extra parts. Again, at this level, implants from segments 10-11 produced both heads and tails.

At posterior levels implants from both anterior and posterior sources produced only tail buds, which in the majority of cases were complete. Similar results were obtained with pieces from the mid-body region.

These results seem to be best explained on the basis of the following theory. There is a strong tail-determining factor in the posterior part of the body. Whenever this is present at full strength, any well-differentiated bud formed will terminate in at least a partial anal segment. This factor is weak or lacking in the anterior part of the body. There is also a head-determining factor, present in the anterior part of the body but either absent in the posterior part of the body or overpowered there by the much stronger tail-determining factor. In the mid-body region (segments 9-11) there is a transition between these conditions found at anterior and posterior levels. This transition may in some way be associated with a general change in the body at the 9-10 intersegmental level. At this point there is a sharp line of demarcation anterior

to which the integument is thicker and richer in glands and the longitudinal muscle bundles are much larger than in the region posterior to this level. No doubt there also exist other, less obvious differences which may be related to the determination of regenerating or induced buds.

That the tail-determining factor at maximum strength is distinctly stronger than the head-determining factor at its maximum is shown by two types of experiments. Implants from the anterior, head-forming region, inserted in the posterior tail-forming part of the host, induce only tail buds, never heads. Implants from the posterior region, when inserted in the anterior part of hosts, also induce only tail buds.

It seems advisable to leave any further discussion of the significance of these results, using implants from the several regions of intact donors, until after presentation of data from experiments already completed using regenerating worms as donors or hosts. Reports on this work with regenerates are now being prepared for publication. An abstract (Sayles, 1941) covering part of the material has already appeared.

SUMMARY

The data presented in the present paper and in the two related papers previously published (Sayles, 1940*a* and *b*) may be summarized as follows.

When oriented with their anterior ends out: (1) implants which include the eighth or more anterior segments will produce definitive head or tail buds with the type dependent to a considerable extent upon which segment of the host is involved; (2) implants including only segment 9 or segments 9–10 will also produce both general types of buds but probably not as frequently as implants from more anterior sources; (3) implants from segment 10 or segments 10–11 occasionally form a head or tail bud; (4) posterior implants which do not include any material from a level anterior to segment 11 very rarely, if ever, produce determinate buds.

When oriented with their posterior ends out: (1) anterior implants involving no cord posterior to the ninth segment produce no head or tail buds; (2) implants including only segment 10 or segments 9–10 rarely give rise to these buds; (3) implants including only segment 11 or segments 10–11 give rise to head and tail buds in many cases; (4) posterior implants including only segment 11 or more posterior segments produce tail buds frequently, but head buds rarely, if ever.

These data indicate that: (1) There is a tail-determining factor which is strong in the posterior half of the body but weak or absent at the

level of segments 1-8. (2) There is a head-determining factor which is strongest in the anterior region of the body, weaker in the mid-body region, and perhaps finally absent in the posterior part of the worm. (3) The tail-determining factor at maximum strength is distinctly stronger than any head-determining factor in *Clymenella*.

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CROSS- AND SELF-FERTILIZATION IN THE ASCIDIAN STYELA

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In 1904 I made a number of cross- and self-inseminations of *Cynthia partita* (*Styela*) at Woods Hole. Single isolated individuals gave the following percentages of self-fertilization: 33, 10, 100, 95, 95, 75, 10, 30, 30, 10, 1, 75, 90, 85, 33, 0, 4, 4, 0, 12, 4. Many other individuals did not spawn. The results ranged from perfect self-fertility to absolute self-sterility. Several experiments were also made with eggs and sperm artificially removed. There was less selfing and not very much cross-fertilization. Owing to the relatively large amount of body fluids that comes over with the eggs, and the large number of immature eggs, the method is inadequate for the best results. This applies also to other tests in which ether, alcohol and ammonia were added to the mixture of eggs and sperm. In most cases more eggs were selfed than in the controls in sea water. It may be noted that when these substances were later used on *Ciona* eggs, they likewise gave erratic results of doubtful significance; but later (1939) it was found that treatment of the eggs with a dilute solution of HCl for 2 to 5 minutes, after washing in sea water and selfing, gave consistently 100 per cent normal cleavage and often normal embryos. I found that the same HCl solutions did not increase the amount of self-fertilization in *Styela*; but it is not improbable, I think, that self-fertilization could be brought about by HCl here also if somewhat different treatment were followed.

In 1930 Plough reported for *Styela* (*Cynthia*) that no selfing occurs in some individuals while other individuals show almost 100 per cent self-fertility. The amount of self-fertilization was determined by a "count of the tadpoles—usually not yet hatched—which were present on the following morning." These counts generally exceeded the number of eggs that had early cleaved. "Thus the full number of eggs which develop was secured, and abnormal cleavages eliminated." In other words, the data do not give the actual number of eggs fertilized but only those that gave normal tadpoles. In the experiments he reported, one lot of eggs (A) was completely self-sterile, while another

lot (K) gave 42 per cent self-fertilization. Cross-fertilization of (A) gave 16, 20, 17, 46 per cent (not 56 as stated in the text), while cross-fertilization of (K) gave 40, 69, 42, 42 per cent.

Plough reported that by adding 4 drops of N/10 NH_4OH to samples of the above eggs the percentage of selfing for K rose to 77 per cent, and the cross lot Ka rose from 36 to 79 per cent.

During the following summer, 1931, I repeated Plough's method with NH_4OH on a rather large scale and failed to get any evidence that self-fertilization was increased in *Styela*.

In 1932 Plough repeated and extended his former experiment and found that most frequently the treated eggs gave a slightly reduced percentage of fertilization both in selfed and crossed samples. The difference between the earlier and later results he explains as due to the eggs of the former record having lost their resistance to their own sperm by standing in sea water before fertilization, since there was an interval of 45 minutes that "elapsed between the fertilization of the first set of egg samples (K) and that of the second (K') in which self sterility was found to have disappeared. In this year's tests the egg samples were prepared at the same time and fertilized almost simultaneously." It should be noted, however, that self-sterility in (K') had not "disappeared" but only 79 per cent of the eggs selfed which was, however, about the same percentage as those that crossed. That more eggs were selfed than those observed to cleave in the normal time is a fact that I have also found in *Ciona*. Frequently the number of tadpoles that appear in a dish of selfed eggs is greater than the number of cleavages recorded after one hour. It is not improbable that some of the increase is due to loss of resistance to selfing on standing, but there is another possibility that cannot be overlooked. There may be present in the sperm suspensions a relatively smaller number of sperm that differ genetically from the majority, and it may take time for them to meet the eggs and fertilize them. This is consistent with the evidence for *Ciona* that the greater the initial amount of sperm, the larger the number of eggs that are self-fertilized. The same relation was noted by Plough. Now in *Styela* the eggs and sperm are set free at the same time, and on removing some of the eggs to another dish of sea water some sperm will as a rule go over with them. If the sperm suspension carries over some of the exceptional spermatozoa, a certain amount or all of it will be used up for self-fertilization, but if more sperm is added either at the time or later, a larger number should be fertilized. The increased number of self-fertilizations that takes place after a time can be explained on this assumption.

Plough points out further that certain of his experiments "suggest that cross fertility may increase during the first three hours after shedding although much less rapidly than self-fertility." He tested this as follows. All of the eggs were removed from dishes into which they were shed and squirted into a larger amount of fresh sea water. "This treatment brings about very considerable dilution of the suspension of own sperm surrounding the eggs and a truer picture of the effect of standing on cross fertility may be expected." He found that selfing increased during two hours but cross-fertility gradually diminished and later rapidly fell off to zero. These results are also consistent with the suggestion that I have made above. Cross-fertilization decreases on standing because the sperm (not the eggs) rapidly lose their fertilizing power. This would be expected also for the selfed lots, but by diluting the sperm it would take a longer time for the relatively fewer number of sperm that can enter their own eggs to reach them. In Plough's table to illustrate his hypothesis there was an increase of 7 to 24 per cent in the selfed lots, i.e., to the extent of three times the amount of selfing.

In 1932 Plough reported at the Sixth Genetic Congress but no new data are given. In 1933 he reported that during the past three summers he and Miss Alderman have been studying *Styela* and have found that decrease and increase of the hydrogen concentration of the sea water, etc., . . . "only reduce fertility all along the line." By the use of crab digestive fluid, the membrane was removed. "There is always a general effect when this fluid is used to digest off the membranes but in the best cases many eggs develop. Strangely enough, removal or weakening of the membrane has no effect on the relation of self and cross fertilization. The membranes appear to have nothing to do with the block to self fertilization in *Styela*." No data are given. He reports that, in sections of selfed eggs, many sperm are found inside the membrane and test cells, and in close contact with the egg cortex, although self-fertilization has not taken place. "Thus self-sterility in *Styela* seems to be a true case of selective fertilization." It should be recalled that the only spermatozoa that normally fertilize the eggs of *Styela*, as shown by Conklin, are at the antipole, hence, even if spermatozoa are present at the surface of the egg, they would not necessarily be expected to enter the egg. It is conceivable, too, that passage through the egg membrane and test cells would render the sperm incapable of entering the egg except those that enter at the antipole. In 1935 Plough found that *Styela plicata* and *S. barnharti* give the same results as *S. partita*. Eggs exposed to their own sperm show that the sperms penetrate the membrane and come into contact with the eggs. "The data suggest that self

TABLE I

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
52	69	0	45	1/2
0	2	22	200	1/4
502	2	0	0	all
323 (mostly eggs)	13	0	3 (one abn.)	all

sterility in *Styela* results from an individually specific blood block to fertilization by the animal's own sperm." No details are given.

During the summer of 1941 I made some further experiments with *Styela* at Woods Hole. The surface of each was scrubbed clean and the animals were kept in a dish with running sea water until 5 or 6 p.m. when they were put each into a dish ("finger-bowls") of sea water (about 200 cc.) and watched from 8 to 10 p.m. in order to record those that laid eggs. As a rule, only a few of the isolated animals set free sperm and eggs. The animals were then put back together into a large dish of running sea water, and the next day, and sometimes on a third day, were again tested for spawning. The dishes in which eggs and sperm had been set free were examined the next morning and the number of unfertilized eggs, of normal swimming tadpoles, of abnormal tadpoles and of abnormal embryos were recorded. It is difficult sometimes to distinguish between the unsegmented (unfertilized) eggs and the abnormal embryos that had only passed through the segmentation stages, but most of the abnormal embryos show a black "eye spot" that clearly indicates they had begun to develop, but had not passed into later stages. As an example of the relative number of those that set free eggs and sperm on one occasion, 14 were tested on July 23-24. Only four spawned. The animals had been brought in July 21. The record of the four that shed eggs and sperm was as follows (see Table I). The number of the eggs, embryos, etc., actually counted is indicated by the fraction in the last column.

TABLE II

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
364	0	0	0	3/4
0	0	0	149	all
0	37	17	510	4/5
0	59	0	37	?

Individuals of the same lot were put out again (July 24-25). Of these 25 did not lay, but one gave 958 eggs, 7 tadpoles and 1 abnormal tadpole. There were also a few abnormal embryos.

Eighteen of the same lot were set out the next day (July 25). The old *Styela*s were left in the dish. Only four spawned. The results are given in Table II.

A fresh lot was brought in July 26 and set out (July 27). The *Styela* remained over night in the dishes. No spawning took place in 25 dishes; in eight dishes spawning took place. The results are given in Table III.

Thirty-three, including the same individuals, were set out July 28, and 16 of them set free eggs and sperm. The old *Styela* had been removed from the dishes. The results are given in Table IV.

TABLE III

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
691	5	0	6	2/3
404	23	64	265	1/3
49	23	64	153	1/4
479	2	0	2	1/2
79	354	23	434	9/10
709	0	0	0	1/8
96	0	0	0	9/10
253	9	16	55	1/4

In addition to the preceding records, there were 19 other individuals that spawned, but since they gave the same kinds of results, it does not seem necessary to record them here.

Taking the last four tables together, there are 25 cases in which some or all of the eggs were selfed. The ratios of unfertilized to those that were fertilized, including tadpoles and abnormal embryos, varied widely. At one extreme (eight cases), all were self-fertilized; at the other (24 cases), some or many eggs were not self-fertilized.

In practically every case in which the number of fertilized eggs is sufficient to give a significant figure, there was a relatively large percentage of the eggs which did not give normal tadpoles. It is not possible to compare these percentages with those reported by Plough, since he states that he counted only the normal (?) tadpoles that had not yet hatched. It is true that it is possible to record the normal tadpoles with long coiled tails just before hatching, but obviously this in most cases

does not give the percentage of fertilization, whether self- or cross-, but only the percentage of normal development.

To what extent leaving the old *Styela* in the dish may affect the development, since it would be passing water in and out of its siphons, was looked into by comparing sets of dishes in which the *Styela* had been left in over night with those in which they had been removed as in the preceding tables.

In the following test (see Table V) the old *Cynthias* had been left in the dishes.

In a later test (August 23-24) forty dishes were put out (5 p.m.) and the old *Styela* left in each dish until 10 a.m. the next morning. In 19

TABLE IV

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
595	5	1	0	2/3
0	92	224	672	1/4
some eggs	38	40	most abn.	1/6
259	12	0	0	3/4
103	3	0	92	1/2
528	0	0	0	1/5
41	12	26	109	1/5
423	0	0	0	1/8
405	24	100	29	1/10
312	5	0	4	1/4
0	11	1427 and abn. embryos		1/10
0	419	92 " "	" "	1/5
325	518	638 " "	" "	1/4
0	29	970 " "	" "	1/10
0	633	21 " "	" "	1/2
rare	302	342 " "	" "	1/5

dishes of 40 set out the individuals had spawned. The record is given in Table VI.

A comparison between these records and those of Table III shows practically no difference in the relative numbers of fertilized eggs, of normal tadpoles and abnormal embryos in dishes (containing about 200 cc. sea water) in which the old *Styelas* were left and in those in which the *Styelas* were removed soon after spawning.

In the next lot of freshly brought-in *Styelas* (July 29) only one individual spawned, giving the following data:

Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos
114	243	108	156

TABLE V

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
382	1	0	0	1/2
302	30	4	0	9/10
700	186	18	25	1/4
493	0	0	1	1/2
361	19	0	0	1/2
601	36	0	17	1/3
273	37	0	9	1/2
475	51	1	31	1/3
354	205	0	34	1/4
241	28	0	7	1/2
508	36	20	87	1/15
2	78	20	87	1/2
324	251	4	18	1/5
555	20	0	7	1/8
87	11	31	333	1/10

The same individuals were tested a day later (July 30). Selfing and reciprocal fertilizations were made between pairs of individuals. Thus *A* eggs were selfed with *a* sperm, and also crossed with *b* sperm. Similarly for *B*. The percentage of self-fertilized eggs (tadpoles and ab-

TABLE VI

Unfertilized Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Counted
433	63	2	8	1/3
294	50	0	10	1/5
6	333	0	0	1/5
74	19	0	179	1/5
111	146	2	106	1/4
34	60	10	439	1/4
503	91	0	3	1/2
197	73	0	9	2/3
0	47	96	128	1/5
118	120	45	25	1/8
286	85	0	7	1/7
135	40	0	10	1/3
297	0	0	0	1/8
33	179	0	51	1/5
53	66	0	87	1/3
357	120	0	—	1/8
202	89	7	23	1/2
169	147	—	26	1/2
9	87	1	2	1/1

normal tadpoles and abnormal eggs) is given in the fifth column of Table VII and the proportion of the eggs counted is given in the last column (approximately).

The percentages of eggs that were self-fertilized varied from 1 to 100 per cent. All the eggs that were cross-fertilized gave 100 per cent both ways, and gave practically 100 per cent normal tadpoles. In con-

TABLE VII

	Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Percentage Fertilized	Counted
{ A eggs self	813	98	0	0	10.8	1/4
{ A eggs by b sperm	0	363	0	2		3/4
{ B eggs self	0	171	0	326	100	1/2
{ B eggs by a sperm	0	153	0	0		4/5
{ C eggs self	82	449	0	41	85.7	1/2
{ C eggs by b sperm	0	448	0	0		1/2
{ D eggs self	0	429	68	470	100	1/10
{ D eggs by c sperm	0	628	0	0		1/3
{ E eggs self	97	24	14	539	85.6	1/5
{ E eggs by f sperm	0	135	134	0		1/5
{ F eggs self	5	513	0	23	99.1	1/6
{ F eggs by e sperm	0	161	0	0		1/2
{ G eggs self	0	593	0	14	100	1/5
{ G eggs by f sperm	0	512	0	2		1/3
{ H eggs self	0	545	0	10	100	1/4
{ H eggs by i sperm	0	431	2	0		—
{ I eggs self	261	143	2	0	36	1/2
{ I eggs by h sperm	0	389	1	1		9/10
{ K eggs self	156	99	0	0	39	9/10
{ K eggs by l sperm	0	2	290	0		9/10

trast, three of the selfed lots produced more abnormal embryos than normals, but the other seven produced fewer abnormalities than normals.

The *Styelas* which made the following records (see Table VIII) had been collected August 2. Nine spawned. As soon as this took place some of the eggs, and the sperm (*A*) that went along with them, were put into two dishes of fresh sea water. To one of these some of the sperm suspension of another dish (*B*) was added. The eggs and sperm

that were left in the original dish are designated "selfed." The experiment was made to find out the proportion of eggs fertilized (as judged later by the number of tadpoles and embryos that developed), and also to find out whether the number of selfed eggs in fresh sea water gave a

TABLE VIII

	Eggs	Tadpoles	Abnormal Tadpoles	Abnormal Embryos	Percentage Selfed	Counted	Percentage of abnormal
A eggs selfed	244	62	9	16	26	1/4	28.8
A eggs in fresh sea water	20	14	10	16	67	1/2	53
A eggs by a sperm	1	258	0	81		1/2	
B eggs selfed	19	92	135	572	98	1/15	88
B eggs in fresh sea water	5	50	5	108	97	1/2	69
B eggs by a sperm	0	558	0	33		1/2	
C eggs selfed	47	234	9	411	93	1/2	64
C eggs in fresh sea water	12	43	1	117	93	2/3	83
C eggs by d sperm	0	93	0	210		3/4	
D eggs selfed	287	15	0	103	32	1/3	87
D eggs in fresh sea water	218	23	0	16	15	1/2	41
D eggs by c sperm	2	71	3	149		1/2	
E eggs selfed	868	0	4	4	0.9	1/10	100
E eggs in fresh sea water	528	0	0	0	0.0	1/5	0
E eggs by f sperm	0	29	16	149		1/2	
F eggs selfed	0	156	4	372	100	3/4	70
F eggs in fresh sea water	0	171	6	87	100	7/8	35
F eggs by e sperm	0	333	8	142		1/2	
G eggs selfed	220	10	0	1	4.8	1/2	9
G eggs in fresh sea water	12	1	0	0	7.7	1/1	0
G eggs by h sperm	27	93	1	27		9/10	
H eggs selfed	190	439	37	11	72	1/3	9.8
H eggs in fresh sea water	20	84	4	8	83	9/10	12
H eggs by g sperm	7	172	1	3		1/2	
K eggs selfed	410	37	9	16	13.1	1/3	40
K eggs in fresh sea water	56	1	0	0	1.8	1/1	0
K eggs by i sperm	23	61	2	1		—	

higher percentage of normal tadpoles than those left in the original dish. The data also show whether the cross-fertilized eggs gave rise to more normal tadpoles than do the self-fertilized eggs. Of course, some or many of these eggs may have selfed before the foreign sperm was added.

Cross-fertilizations were made in pairs (see below) for comparison with each other. The percentages of selfing are given in the fifth column; and the percentages of fertilized eggs that developed abnormally are given in the sixth column.

The percentages of eggs that selfed in the original dish, as compared with those that were removed to fresh sea water, are smaller in only three cases. In two cases the percentage was greater but not much greater; in one case nearly the same (98 and 97 per cent), and in another the same (93 and 93 per cent). There was probably much more sperm in the original dish and also many more eggs, but the percentages of the two may be compared. On the whole, the eggs developed as well in the original dish (from which the *Styela* was removed) as in the dish of fresh sea water.

The number of abnormal embryos is quite high in all the dishes. The percentages of abnormals in original and fresh water dishes were: 28.8-53; 88-69; 64-83; 87-41; 100-0; 70-35; 9-0; 9.8-12, 40-0. Rejecting the fifth and ninth pair, since the numbers are too small to be significant, the counts show that there is no consistent relation of normals to abnormals in the two kinds of dishes; sometimes one, sometimes the other is the greater. In other words, the original dishes of eggs and sperm did about as well as those with fresh sea water in them and fewer eggs. The latter contained eggs, some or all of which may have been fertilized before transfer to fresh sea water, but this would not affect the kind of development that took place.

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CROSS- AND SELF-FERTILIZATION IN THE ASCIDIAN MOLGULA MANHATTENSIS

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The rule for the "single" ascidian *Ciona intestinalis* is no self-fertilization and 100 per cent cross-fertilization, but there are rare cases of the reverse and it is these that may give a clue to the genetic problem involved. Another single ascidian, *Cynthia*, has been shown to self-fertilize frequently but no cases of cross-sterility have been reported so far. A third single ascidian, *Molgula manhattensis*, has been shown to give a high percentage of self-fertilization, mainly from my own earlier examination (*Jour. Exper. Zool.*, 1904), but no thorough comparison of the extent of selfing with the extent of crossing has been made. It seemed worth while to undertake such an examination in the hope that some degree, at least, of self-sterility might be found and, if so, fall in line with the situation in the other two species. In my former paper there were cases of 100 per cent self-fertilization and also a few in which no selfing occurred.

During September, 1941, *Molgula* being abundant on the float in the eel pond at Woods Hole, a number of them were tested. There is an ovotestis on one side, in a loop of the intestine. It is smaller than the one on the other side, and its central slate-colored ovarian portion is, as a rule, more nearly surrounded by the white testis than is the ovotestis on the other side, and this makes it more difficult to remove the ripe eggs without contamination from the testis. The larger ovotestis on the opposite side is generally only partly surrounded by the testis, and the eggs can frequently be removed by a small puncture in the ovarian portion without contamination.

Each individual was first washed in tap water, the test was split open, and the interior mass removed and also washed. A small puncture in the ovarian part allows the eggs to exude. They were then collected in a dish of sea water (15 cc.).

In a preliminary test of six individuals (August 28) three lots of eggs gave no cleavage, showing that no sperm had come out with the eggs, or at least not sufficient sperm for selfing. Three sets gave a high percentage of cleavage, which must have been due to undetected sperm that

had come out with the eggs. The three sets of eggs that had not cleaved were cross-fertilized and all cleaved.

In later experiments the eggs were first removed from an individual (*A*) to a Syracuse dish of sea water; one-half of these was transferred to another dish and later cross-fertilized with sperm from the "reciprocal" individual (*B*). More eggs and some sperm were then removed together from the same individual to another dish. The latter were heavily inseminated with their own sperm. At the same time similar dishes were prepared from individual (*B*), some of whose eggs were crossed with sperm from (*A*). If none of the unfertilized eggs in *A* and *B* cleaved, it would mean that no spermatozoa (or too small an amount to be effective) have been introduced into this dish. Hence, if any or all eggs in the cross-fertilized dish cleaved this was due entirely to cross-fertilization. If any or all eggs cleaved in the self-fertilized dish, this must have been due to its own sperm. As an illustration, the results for three such tests were as follows:

- (A) The unfertilized eggs of *A* gave no cleavage; those selfed gave 25 per cent cleavage; those crossed by *B* gave 100 per cent.
- (B) The unfertilized eggs of *B* gave no cleavage; those selfed gave 100 per cent; those crossed by *A* gave 100 per cent.
- (C) The unfertilized eggs of *C* gave no cleavage; those selfed gave no observed cleavage (but later one coiled embryo was present and a few abnormal embryos); those crossed by *D* gave 100 per cent.
- (D) The unfertilized eggs of *D* gave no cleavage; those selfed gave 1 per cent; those crossed (to *C*) gave 75 per cent.
- (E) The unfertilized eggs of *E* gave 10 per cent cleavage; hence to this extent some of the "cross-inseminated" eggs may have been selfed before foreign sperm was added; the selfed eggs gave 100 per cent; and the crossed eggs (by *E*) gave 100 per cent.
- (F) The unfertilized eggs of *F* gave no cleavage; the selfed eggs gave no cleavage; those crossed to *E* gave 100 per cent.

It is evident from these tests that the amount of selfing that occurred varied from 0 to 100 per cent, while the cross-fertilized eggs gave 100 per cent cleavage except in one case (75 per cent).

Two of the preceding combinations (A-D) are recorded in the first two pairs of Table I. This table gives the results from 38 pairs (or 76 individuals). The respective pairs used in reciprocal crossing are bracketed. With three exceptions the cross-fertilized eggs gave 100 per cent cleavage. The three exceptions (75, 50, 80) were in different pairs. There was nothing peculiar in the selfing of these three cases unless it be

TABLE I

Control	Self	Cross	Control	Self	Cross	Control	Self	Cross
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 25 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 5 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 50 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 1 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 75 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 2 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 75 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 2 \\ 40 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 50 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 90 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 80 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 90 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 35 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} ? \\ 2 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 30 \end{Bmatrix}$	$\begin{Bmatrix} 12 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 10 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 2 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 75 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 50 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 90 \\ 15 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 90 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 80 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 80 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 50 \\ 50 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 50 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 50 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 4 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 8 \\ 8 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
$\begin{Bmatrix} 20 \\ 4 \end{Bmatrix}$	$\begin{Bmatrix} 20 \\ 14 \end{Bmatrix}$	$\begin{Bmatrix} ? \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 15 \\ 70 \end{Bmatrix}$	$\begin{Bmatrix} 15 \\ 80 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 0 \end{Bmatrix}$	$\begin{Bmatrix} 0 \\ 20 \end{Bmatrix}$	$\begin{Bmatrix} 100 \\ 100 \end{Bmatrix}$
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that the 50 per cent crossed eggs gave 100 per cent selfed. In addition there were two cases in which, by oversight, the crossed eggs were not recorded, but there can be no doubt that they gave 100 per cent cleavage or attention would have been drawn to them.

An examination of this table shows that no cleavage took place in 44 lots of eggs removed from the ovary and not inseminated. There were 32 other lots with percentages ranging from 1 to 100 per cent selfing. This means that the amount of sperm that had been unintentionally re-

moved with the eggs was too small in amount in most cases to self-fertilize any of the eggs. An examination of the table shows that, in cases where no cleavage occurred in the dishes of control eggs, it also did not take place in 9 of the selfed dishes, which must mean that 10 per cent of the individuals were totally incapable of self-fertilization. Of those lots in which none of the control eggs cleaved (44 cases), and some eggs at least cleaved when selfed, the percentage of eggs cleaved ranged from 2 to 100 per cent. This may be interpreted to mean that although plenty of spermatozoa were present, only a percentage of them were actually effective as measured by the cleavage. I interpret this to mean that in addition to the spermatozoa having the same genetic composition as the eggs, there are varying percentages of sperm with a different genetic composition, and these are able to fertilize eggs from the same individual. This interpretation is also consistent with the evidence from those cases where a small percentage of the eggs in the control uninseminated dishes cleaved. As the table shows, there were 20 cases in which the control "unfertilized" eggs that cleaved gave a lower percentage of cleavage than did the "self-fertilized" eggs, and only 10 cases in which they were the same. These results are consistent with the assumption above, viz., that there are present a smaller and variable number of spermatozoa whose genetic make-up differs from the majority of sperm, the latter incapable of fertilizing their own eggs, but the former having the ability to do so. This conclusion is consistent with the more completely worked-out case of *Ciona*.

It should be noted that I did not come across a single case of cross-sterility, but the numbers may have been too small to expect to meet such cases. There is one case recorded where no eggs were selfed and only two eggs cleaved when cross-fertilized, but I am inclined to think that the sperm was not in condition to self or to cross. It is noticeable here that there is a question mark in the table when the sperm of this individual was used for crossing the reciprocal.

NORMAL AND ABNORMAL DEVELOPMENT

In some of the preceding experiments the dishes were examined when the tadpoles were due to hatch (or after that time) in order to find out whether the development was normal or abnormal. The observations are not here reported since it became apparent that unless the embryos are followed closely before and at the time of expected hatching and later, the records of what constitutes abnormals cannot be accurately defined. For instance, the tadpoles are due to emerge ten to twelve hours after fertilization depending on temperature. Besides being a

rather inconvenient hour for taking records, many of the embryos without passing through a free swimming stage undergo metamorphosis in place, and the time may extend over a considerable interval. This makes it difficult to record normal versus abnormal embryos. In general, it may be said that in a large number of cases there were many abnormal embryos as compared with the number of those that cleaved. This applies mainly to embryos that did not pass into the complete tadpole stage. Those that underwent a metamorphosis without passing through the free-swimming tadpole stage were so variable that without fuller records it is not possible to make a clear statement about them. Further observations are needed. There was no evidence that the selfed lots behaved differently from the cross-fertilized lots.

TWO UNISEXUAL MOLGULAS

* Two individuals were found that had no testis on either side but well-developed ovaries. These came from a small bunch of some 35 to 50 individuals, and may therefore have been related. They were full-sized individuals and normal in all other respects. Eggs were taken out separately from each side of each individual. One-half of the eggs of each were kept in case undetected sperm was present, but none of them cleaved. The cross-fertilized eggs taken from one side of one individual cleaved irregularly. Later they gave rise to a few tadpoles and some metamorphosing embryos. The eggs from the other side when crossed gave abnormal cleavage (polyspermic?) and later a few metamorphosed individuals. Eggs taken from the other individual and not fertilized did not cleave. Those from one side that were cross-fertilized gave 100 per cent cleavage and later many metamorphosed individuals. Those from the other side that were fertilized gave 90 per cent cleavage and later a few normal tadpoles and many metamorphosed embryos. The eggs of these two individuals when cross-fertilized did not behave differently from those of bisexual *Molgulas*. If a new generation could be reared from such unisexual forms, it might be possible to find out if the condition is inherited.

SUMMARY

Tests of self- and cross-fertilizations of the single ascidian *Molgula manhattensis*, were made during August and September, 1941, at Woods Hole. It was found that 100 per cent self-fertilization occurred in less than half the cases (see Table I), but in others the percentages ranged from 1 to 90 per cent. One hundred per cent of cross-fertilization oc-

curred in practically all cases. The occurrence of normal and abnormal development was observed, and many cases of abnormal embryos as well as normal tadpoles were found, but, owing to the fact that many embryos metamorphosed directly, or at least without passing through a free-swimming tadpole stage, the numerical results are not reported here. A closer set of observations are needed to give reliable data as to whether self-fertilized eggs give rise to such development more often than do cross-fertilized eggs. Two mature individuals were found that had only ovaries. Their eggs gave 100 per cent cross-fertilization.

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THE APPLICATION OF THE PRECIPITIN TECHNIQUE TO THEORIES CONCERNING THE ORIGIN OF VERTEBRATES

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INTRODUCTION

Various types of evidence have been employed to determine the relationships between chordates and the different invertebrate groups, and numerous theories have been advanced to derive vertebrates from echinoderms, annelids, and arthropods. These theories have been based on subjective considerations and have resulted from individual interpretations of the value of particular structures in determining descent. At the present time, most workers are agreed that theories which derive chordates from echinoderms are probably correct. However, as recently as 1922, Delsman supported the annelid hypothesis of vertebrate ancestry. The consensus of present-day opinion regarding vertebrate origin is represented in Allee's diphyletic tree which shows probable relationships of the principal groups of the animal kingdom. The principal evidence for this scheme is embryological: (1) the fate of the blastopore which in both echinoderms and chordates does not become the mouth but forms the anus or a nearby structure (neurenteric canal), placing these animals in the group known as Deuterostomia, and which in annelids, arthropods and molluscs becomes the mouth, placing these animals in the group known as Proterostomia; (2) the method of mesoderm and coelom formation, which in deuterostomians is by paired evaginations from the endoderm, forming enterocoelous pouches, and which in proterostomians is by proliferation from two mesoblastic or pole cells, the mesodermal bands later splitting and cavitating to form schizocoelous spaces; and (3) the type of cleavage, which in echinoderms and chordates is, generally speaking, indeterminant, and in annelids, arthropods and molluscs is determinant.

Biochemical confirmation of ideas which derive chordates from echinoderms has come from an analysis of their phosphagens, reported by Needham et al (1932). These substances, so important as sources

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of immediate energy during muscular contraction, are present as creatine phosphoric acid in vertebrates and as arginine phosphoric acid in invertebrates. However, in the sea urchin, *Strongylocentrotus lividus*, and the prochordate, *Balanoglossus salmoneus*, these two phosphagens occur together indicating that the invertebrate showing closest chemical affinity to the prochordate is the echinoderm.

In view of the existing controversy, an objective test which can reveal and express quantitatively the relationships between organisms is desirable in the solution of this problem. In interrelationship studies, morphological and developmental data have been supplemented in recent years by the results obtained from serological reactions. As indicated in a number of researches, the precipitin test is best suited for determining interrelationships of organisms, since it "lends itself admirably to qualitative, quantitative, and *objective* interpretations, which are necessary and fundamental for the evaluation of serological relationships" (Wilhelmi, 1940).

Though never before intensively studied, it seemed that analysis of precipitin reactions between materials of various invertebrates and prochordates would indicate which invertebrate group has the closest physiological and chemical affinity, and presumably, therefore, the closest genetic relationship, to the chordates. This information provides an objective basis for establishing the origin of vertebrates. Accordingly, the purposes of the present investigation are to determine: (1) species-specific titer, thus giving a reference value for the heterologous titers which might be obtained, (2) interrelationships of the several species of invertebrates and prochordates, and (3) relationships between the species of invertebrates and between the species of prochordates.

MATERIALS AND METHODS

Since three phyla of invertebrates have been involved in theories of vertebrate origin, several species from each phylum (except Arthropoda) were selected, in addition to several species of prochordates, for the preparation of antigens. The following species were employed: ECHINODERMATA—*Asterias forbesi*, *Arbacia punctulata*, *Echinarachnius parma*, *Leptosynapta inhaerens*, *Thyone briareus*; ANNELIDA—*Arabella opalina*, *Chaetopterus pergamentaceus*, *Cirratulus grandis*, *Glycera dibranchiata*, *Phascolosoma gouldi*; ARTHROPODA—*Limulus polyphemus*; CHORDATA—*Dolichoglossus kowalevskyi*, *Amaroucium constellatum*, *Molgula manhattensis*, *Styela partita*. It will be noted that the habitat of all these animals is marine; hence, species differences are expressions of genetic differences, and those resulting from environmental influences are reduced to a minimum.

Preparation of Antigen

When the whole serum of an animal is employed, the preparation of antigen becomes a simple matter of removing the blood, allowing it to clot, and filtering the serum which separates. However, when the sources are invertebrates, and especially the lower phyla which have no blood, all antigens are necessarily extracts of tissues, and, hence, numerous difficulties are encountered and many precautions must be exercised.

To remove certain contaminating organisms, foreign proteins and debris, the animals when obtained were kept in large crystallizing dishes supplied for a day or so with filtered fresh sea water. Following the preliminary period of exposure to filtered sea water, they were carefully washed through several changes of sterile sea water contained in covered crystallizing dishes, and finally quickly through sterile distilled water to effect a removal of excess salts. This careful washing procedure reduced the bacterial contamination to a minimum, and the long period of washing allowed elimination of fecal material from the alimentary tract, although the alimentary tract was removed in most cases, especially in the annelids. Manipulations in and through the sterile media were done carefully to avoid contamination, sterile forceps being used to effect transfers from one dish of sterile medium to another.

Having reduced contamination, the soft tissues of the living animals were minced on a glass plate or in a shallow glass dish (Petri) and frozen rapidly, using dry ice. Material, once frozen, was kept at this low temperature in a vacuum desiccator (evacuated by a Cenco-Hyvac pump) over anhydrous CaCl_2 or $\text{Mg}(\text{ClO}_4)_2$ until thoroughly dried. As noted in an earlier paper (Wilhelmi, 1940), this freezing-drying, or so-called lyophile, process has certain obvious advantages. Rapid freezing preserves the original structure of labile substances, and the high vacuum causes rapid dehydration and—more important—autorefrigeration to the extent of keeping the materials frozen. Furthermore, the process removes oxygen which could act on antigenic substances to alter their native structure. Desiccation in the frozen state results in dried material which has a spongy texture, and such material is readily pulverized.

After twenty-four hours in the vacuum, the material was pulverized and then returned to the desiccator to insure complete desiccation, indicated when a constant weight was attained. Certain of the annelid materials required a longer time for complete desiccation.

The lipids were removed from the material by repeated extractions with Bloor's mixture, consisting of three parts absolute ether to one part absolute alcohol.

After weighing the lipid-free residue, it was extracted for twelve to twenty-four hours with buffered saline solution maintaining a pH of 7.3, extraction being effected in Erlenmeyer flasks at room temperatures and under constant agitation provided by a mechanical shaker.

After aqueous extraction the material was filtered through sterilized fritted Jena-glass filters, and the clear, sterile filtrate was transferred to sterile, cotton-plugged, small Erlenmeyer flasks and kept in the refrigerator until needed. The residue was thoroughly desiccated and weighed, and the exact amount of material in solution was determined by calculating the difference in weight of residues before and after the aqueous extraction.

Antibody Production

For injection purposes, rabbits of equivalent weights were used after an observational period of several weeks prior to experimentation. Four intravenous injections, one every third day, with gradually increasing dosage, had been found earlier (Wilhelmi, 1940) to be the most satisfactory procedure in the rapid production of high-titered, consistent antisera. This short method of antibody production was followed in the present experiments, but the incubation period following the last injection was increased to twelve or fifteen days.

In the series of four injections, a total of 500 mg. of dry-weight, lipid-free antigen was injected. As in the experiments noted above, this total was divided into doses of increasing amounts, i.e., 25, 75, 150 and 250 mg., respectively, in these series.

After the period of incubation, the blood, withdrawn aseptically by intracardial puncture, using a large, sterile hypodermic syringe fitted with a 19-gauge needle, was placed in sterile, cotton-plugged centrifuge tubes or Erlenmeyer flasks and allowed to clot. After standing in the refrigerator for several hours, the serum was drawn or poured off, usually subsequent to centrifugation. In cases where more than one rabbit was employed in the production of antiserum to a single antigen, the resultant antisera were pooled; the species involved were *Arbacia punctulata*, *Chaetopterus pergamentaceus*, and *Limulus polyphemus*. Antisera were used immediately for tests or placed in sterile bottles for storage in the refrigerator until needed for the precipitin reaction.

Precipitin Test

In a precipitin tube (8.0 mm., inside diameter), 0.5 cc. of undiluted antiserum was carefully overlaid by 0.5 cc. of appropriately and variously diluted antigen so that a definite interfacial boundary between the

two reagents was maintained. The antigen was brought to the proper concentration by adding sterile saline solution buffered to a pH of 7.3. A standard dilution of each antigen was prepared so that 1.0 gram of dry-weight, lipid-free material would be contained in 100 cc. of saline solution, i.e., 0.01 g./cc. or a dilution of 1:100. In homologous tests this standard was diluted to 1:1000 for the initial tube, and the dilution in each succeeding tube of a series was doubled, so that a series of antigen dilutions from 1:1000, through 1:2000, 1:4000, etc. up to 1:4,096,000 was employed. In the heterologous tests, dilutions from 1:100, or in some cases, 1:50, were used in the initial tube, and the concentration of antigen was halved in each successive tube. In a positive reaction, a ring or layer of white precipitate occurred at the interphase of antiserum and antigen.

The titer of a reaction is the highest antigen dilution which yields a visible precipitate within one hour at 37° C. when tested with an antiserum, either homologous or heterologous, the amount of antigen being determined on the basis of dry weight of antigenic material actually in solution.

Appropriate control tests were always conducted and involved the use of (1) normal sera and antigen solutions, (2) immune sera and the extracting salt solution, and (3) normal sera and the extractant.

OBSERVATIONS AND RESULTS

Table I presents the titers of both homologous and heterologous reactions between the several species of invertebrates and prochordates employed in these experiments. Each titer represents the average of two, or, in many cases, three determinations at 37° C. Negative reactions are indicated by minus signs (—), and tests which were not performed, by blank spaces.

Although rather high, titers of homologous tests cover a wide range, from 1:64,000 to 1:2,048,000. However, for the most part, the species-specific titer is 1:1,024,000 plus or minus one dilution tube. Marked deviations from this value are the titers for *Thyone briareus* among the Echinodermata, and *Arabella opalina* and *Cirratulus grandis* among the Annelida, and, of these, the deviations can be explained in all except the *T. briareus* materials. *Amaroucium constellatum* materials were tested, but a relatively low homologous titer (1:32,000) was obtained and the results are not included here since the low titer is explained by crystallization which occurred in the flask containing the antigen, after the injection series had been started; the tautomeric crystallization apparently removed enough antigenic material from solution

so that not only was considerably less than 500 mg. injected but also the dilution in the last tube yielding a positive reaction was probably greater than 1:32,000.

Wherever available materials permitted, reciprocal tests were conducted, and, in general, such tests confirmed the original ones, i.e., were of the same order of magnitude. Such confirmation is imperative if the data and resultant interpretations are to be significant. As Boyden (1926) so very appropriately stated, "it would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. . . . Only those values which check within the limits of error of the reaction may then be taken."

Titers of cross-reactions between members of the various invertebrate phyla and the Prochordata were, in general, much higher in the case of echinoderm-prochordate reactions than in annelid-prochordate and/or arthropod-prochordate tests. The titers of heterologous reactions between annelid and arthropod materials were of the same order of magnitude as those between echinoderm and prochordate materials.

In Table I, the figures in parentheses represent the titration data converted to percentages. As in reports by other authors, the homologous titer of a particular antiserum is taken as 100 per cent and the titers of all heterologous tests with that antiserum are expressed as percentages of the homologous titers. For example, *Leptosynapta inhaerens* antigen when tested against *Dolichoglossus kowalevskyi* antiserum yields a titer of 1:8000; the homologous titer of *D. kowalevskyi* antiserum being 1:512,000, the percentage value becomes $\frac{8000}{512,000}$ or 1.562 per cent.

DISCUSSION

Homologous serological reactions of high titer would be necessary if heterologous reactions between such distantly related groups as prochordates and any of the invertebrate phyla are to be obtained. Hence, large amounts of antigen were injected in the hope that a maximum antibody response of the rabbit would be elicited, and, from the results, it is apparent that a strong, though perhaps not maximum, antibody response was evoked. With three exceptions, the titer of homologous reactions was 1:1,024,000 plus or minus one dilution tube. No explanation is apparent for the aberrant titer of 1:256,000 obtained with *Thyone briareus* materials. However, in both *Arabella opalina* and *Cirratulus grandis*, the lower titers seem related to the difficulty experienced in desiccating the animals; at least, there is a possibility that antigenic materials

were altered because desiccation required two or three days, as compared with the usual eighteen to twenty-four hours, and the worms did not remain frozen until dehydrated. Of course, individual variations in the ability of rabbits to produce antibodies may be sufficient to cause these variations in titers, but the rabbits used were progeny of a single pair and were of equivalent weights, which would seem to reduce the variability as a result of genetic factors.

In spite of the wide range in homologous titers, the results in the present work are rather uniform, when compared with those reported in certain other investigations, since the exceptions in all but one instance can be satisfactorily explained and the deviation in that one instance is only one dilution tube removed from the generally accepted limit of error. Although the lipids have not been employed as antigens in direct tests, it is probable that extraction of lipids is largely responsible for the uniformity of results. It seems appropriate, therefore, to reiterate and to reemphasize certain observations concerning the specificity of these haptenic components, although a more complete account was presented in an earlier report (Wilhelmi, 1940). While the serological and chemical properties of proteins closely parallel the zoological relationships of organisms from which they are obtained, i.e., the more similar the proteins in organisms, the closer the phylogenetic relationships, the lipids of closely related species, or, indeed, even in different organs of the same species, may differ considerably, and the lipids of distantly related species may be very similar, if not identical, immunochemically. Boyden's (1936) observation that "*the convergent reactions obtained with antisera to native serum . . . disappeared following ether extraction*" and results from the present experiments demonstrate that the group-specific or non-specific character of lipids demands their complete removal from antigenic materials designed to determine zoological or phylogenetic relationships of organisms.

Serological methods have been increasingly stressed in experimental work designed to clarify phylogenetic relationships of animals, at least within more or less restricted groups, such as orders, classes, and phyla. Among invertebrate Metazoa, research involving serological reactions and relationships has been rather limited, being confined for the most part to experiments with helminths. Erhardt (1931) reviewed the serological studies which have involved interrelationships of animals, but in very few cases were positive interphylar reactions noted. In Nuttall's (1904) classic treatise, three doubtfully positive reactions between anti-xiphosuran serum and fish serum were reported, as well as a few positive crustacean-amphibian and crustacean-reptilian reactions. Boyden (1934,

1936) has recorded a serological study of the relationships of some invertebrates, but, unfortunately, results in the form of titers, etc. were not noted, although antigens of Nemertinea, Annelida, Mollusca, Arthropoda, Echinodermata, and Chordata had been collected and injected. Boyden (1934) stated, "These tests clearly indicate that significant data regarding the interrelationships of the members of the higher invertebrate phyla may be obtained through the use of the precipitin reaction. They indicate furthermore that interphylar reactions may even be obtained—a point of some phylogenetic importance."

In spite of general agreement, based on morphological and developmental data, that echinoderms and chordates arose from a common ancestral stock, the origin of chordates, and especially of vertebrates, from the invertebrates has been the subject of considerable speculation and theorization. Many zoologists still contend that theories which derive the vertebrates from the Annelida are most plausible; indeed, as noted above, Delsman (1922) published a modified annelid theory to explain the origin of chordates. The results of the heterologous precipitin tests in the present experiments indicate clearly that echinoderms are the invertebrates with the closest chemical and physiological affinity to prochordates. The titers of echinoderm-prochordate tests are several times greater than the highest titer exhibited by annelid-prochordate or arthropod-prochordate reactions, being, in some cases, twenty times the titer of the proterostomian-prochordate tests, i.e., five dilution tubes removed from the latter. It is noteworthy, however, that the materials of Annelida and Arthropoda do react with those of Prochordata, even if at very low titers.

Results available at present indicate that, of the echinoderms tested, the holothurians are most closely related to the prochordates, and, of the latter group, to the hemichordates, since the *Leptosynapta inhaerens-Dolichoglossus kowalevskyi* reactions yield the highest interphylar titers, both the original tests and the confirmatory reciprocals. However, more tests are necessary before a final appraisal or judgment can be given.

If the Proterostomia separated from the Deuterostomia early in the evolutionary history of animals, then one should expect cross-reactions to occur between annelid and arthropod materials at higher antigen dilutions than between either of these and the deuterostomian materials. Such higher titers were obtained, thus speaking for the closer phylogenetic relationship between Annelida and Arthropoda than between either phylum and the Deuterostomia.

Intraphylar heterologous tests were made for purposes of comparison with the interphylar reactions. The available results demonstrate much

higher titers in intraphylar than in interphylar reactions. However, relationships within restricted groups were not the primary interest in these experiments, and, furthermore, lack of materials has prevented an extension of the researches to include more intraphylar tests. These preliminary results will have to be supplemented before the results of precipitin tests can be applied to phylogeneses within the restricted groups.

When the experimental results are converted to percentages, many of the differences are reduced and many of the discrepancies disappear completely. For example, the titer of the reaction between *Arbacia punctulata* antigen and *Echinarachnius parma* antiserum was 1:32,000, while in the reciprocal tests the titer was 1:128,000; yet, the resultant percentage values are 6.25 in both instances. Also, as may have been noted, the titers of reactions involving *Styela partita* antigen and *Leptosynapta inhaerens* antiserum (1:1000) and *S. partita* antigen and *Limulus polyphemus* antiserum (1:400) are rather close (a difference of slightly more than one dilution tube), although the percentages are very different, the prochordate-echinoderm value (0.098) being five times greater than the prochordate-arthropod value (0.0195). One of the most striking interpretations derived from percentage data is the enhancement given to the suggestion that holothurians (Echinodermata) and hemichordates (Prochordata) are most closely related serologically; indeed, the percentage values between these groups are at least twice as great as the nearest echinoid-prochordate or asteroid-prochordate value. Treating titration data on a percentage basis is necessary in attempts to compare and to correlate experimental results reported by authors whose techniques and procedures have differed. However, in spite of the rather general use of percentages and in spite of the enhancement given to certain suggestions and interpretations derived from analysis of the titers in the present work, it seems advisable to analyze and interpret the results of experiments directly from titration data, and, in future research, to attempt the elimination of discrepancies between titers of comparable reactions by further refinements in technique.

SUMMARY AND CONCLUSIONS

The precipitin reaction, demonstrated to be valuable in determining phylogenetic relationships of animals within restricted groups, was applied to distantly related groups among the invertebrates and chordates to test, by this objective method, the various theories concerning the origin of vertebrates. From the results it is apparent that serologically, as well as morphologically and developmentally, the chordates are more closely related to the echinoderms than to any other group of inverte-

brates tested, indicating that chordates evolved from animals which also gave rise to present-day echinoderms.

Consistent results, confirmed by reciprocal tests, were obtained by injection of aqueous solutions containing five hundred milligrams of dried antigenic material from which the lipids had been removed. The rather uniform results may be attributed to the following important steps: vacuum desiccation of materials in the frozen state (lyophilization), preliminary removal of lipids from desiccated material to exclude the possibility of group reactions, control of hydrogen ion concentration during extractions and tests, control of temperatures at which reactions are performed, and knowledge of the actual amount of material in the antigenic solutions.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, the amount of antigen being determined on the basis of dry weight of antigenic material actually in solution. With three exceptions, the titer of homologous reactions is 1:1,024,000 plus or minus one dilution tube, and heterologous titers never exceeded homologous ones.

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HISTOLOGY OF THE RADULA PROTRACTOR MUSCLES OF BUSYCON CANALICULATUM LINN.

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The muscle fibers of the molluscs have perhaps been more completely studied than those of any other invertebrate group, yet there is no real agreement on their structure. One explanation for this confusion is the apparent presence in most molluscs of several types of muscle fibers. Roskin (1935) describes six different types of smooth muscle fibers and a primitive type of cross-striated muscle in five closely related species of pteropod molluscs. Marceau (1937) in a study of the adductor muscles of more than twenty bivalve molluscs describes five rather distinct types as well as a number of transition or combination forms of the five types.

Perhaps the most serious confusion in the literature on molluscan muscle has resulted from the dispute as to the presence or absence of cross-striated, obliquely striated, and spirally striated muscle fibers. Schwalbe (1869) believed that the arrangement of granules might give the appearance of striations. Schwalbe, however, did describe a type of double oblique striation in which the contractile substance appeared as a diamond-shaped design. Paneth (1885) stated that the muscles of the heteropods and pteropods were in all essentials like the muscles of the vertebrates. Fol (1888) drew the conclusion that the appearance of cross-striation or the double oblique striation of Schwalbe might result from the spiral twisting of the myofibrils. He believed that the only type of muscle present in the molluscs was smooth.

Merton (1911) found both smooth and striated muscle present in the molluscs, but he considered the striated muscle to be different from that of higher animals since the striations disappeared in the resting state. He also gave several explanations for the manner in which smooth muscle fibers might under certain conditions resemble cross-striated muscle fibers. In the first place, the contracted fiber could be fixed so that the myofibrils occurred as wavy lines. The curve of the fibril would stain heavily, the straight portion lightly and thus give the appearance of cross-striations. In the second place, under certain conditions of fixation and staining, the connective tissue fibers running at right angles to the muscle fibers might be confused with cross-striations. Finally, he uses the ear-

lier explanation of Schwalbe that the granules might be aligned to simulate cross-striations.

Plenk (1922, 1924) states that all of the fibers in the masticating apparatus of the snail are cross-striated although he does not deny the presence of smooth muscle in other parts of the body. He believes that apparently smooth muscle fibers are really cross-striated fibers in a certain functional state and that a much more widespread existence of striated muscle in the animal must be assumed. The oblique and spiral striations described by earlier workers are explained by him as distortions resulting from the rigors of fixation and preparation.

The trend of later investigations (Roskin 1925, 1935, Marceau 1937), seems to be the recognition of the presence of different types of smooth and striated fibers.

For the sake of clarity I have limited the present investigation to a detailed study of the radula protractor muscles of *Busycon*.

MATERIALS AND METHODS

The radula protractor muscles of *Busycon canaliculatum* consist of three pairs of flattened bands on the ventral side of the buccal mass. The median pair are somewhat thicker than those which cover the horns of the odontophoral cartilage. In the resting state the muscles are about 2.5 cm. long. The median pair are about 1.5 mm. wide and 1 mm. thick; the two smaller pair are about 1 mm. wide and .75 mm. thick.

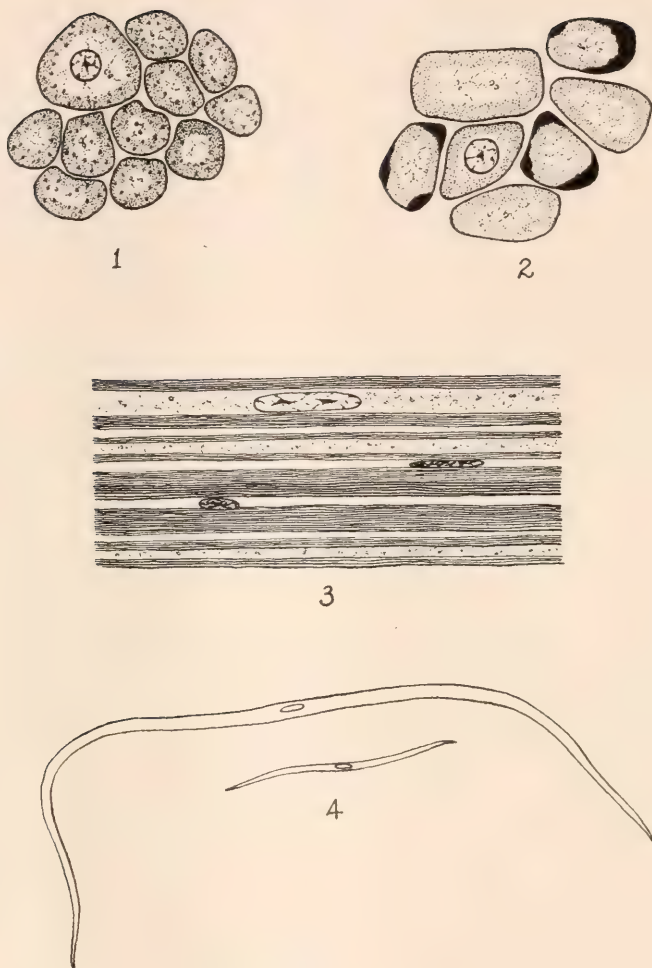
The muscles were fixed in varying states of contraction in Bouin's and Helly's fixing fluids. They were dehydrated in Cellosolve, cleared in xylene, imbedded in paraffin, and sectioned at 5 and 10 micra. The sections were stained with Heidenhain's iron haematoxylin, Mallory's phosphotungstic acid haematoxylin, and Dominici's staining mixture.

The muscle fibers were dissociated with 35 per cent KOH.

RESULTS

The muscles consist essentially of densely packed, spindle-shaped, uninnervated, unstriated fibers which have an average length of about 1 mm. Occasional fibers are found which attain a length of about 1.7 mm. and others which may be as short as 360 micra. The average diameter in contraction is about 6 micra and in extension about 3.5 micra.

In cross-section the fibers appear cylindrical, hemicylindrical, or angular (Figs. 1 and 2). The fibers possess two clearly defined zones; a rather heavily staining cortex and a lightly staining medulla. A number of comparatively coarse myofibrils are scattered throughout the cortical



EXPLANATION OF FIGURES

All drawings were made with the aid of a camera lucida.

FIG. 1. Cross-section of a small portion of an extended muscle stained with Mallory's phosphotungstic acid haematoxylin showing the shape and arrangement of fibers, the fibrillar cortex, and coarsely granular-vesicular axillary region of each fiber. $\times 1750$.

FIG. 2. Cross-section of a small portion of a contracted muscle stained with Heidenhain's iron haematoxylin. The coarse myofibrils are not visible with this stain but areas of contraction stain heavily. $\times 1750$.

FIG. 3. Longitudinal section of a portion of an extended muscle stained with Mallory's phosphotungstic acid haematoxylin showing the fibrillar cortex, the coarsely granular-vesicular medullary portion. The nucleus of a muscle fiber is shown lying in the medullary region and connective tissue nuclei are shown lying between the muscle fibers. $\times 875$.

FIG. 4. A large and a small fiber from a preparation of macerated muscle. $\times 88$.

zone with a slight concentration at the boundary of the cortex and medulla (Figs. 1 and 3). The myofibrillae are neither as fine nor as abundant as those observed in the muscles of *Phascolosoma* (Olson, 1940). The fibrils are visible only with Mallory's phosphotungstic acid haematoxylin, in which respect they differ from those of *Phascolosoma*. With Heidenhain's iron haematoxylin it was possible to see the darkly staining regions of contraction in the cortical zone of the fibers. It is rather peculiar that these are not clearly defined nodes, involving a whole segment of a fiber as is true in *Phascolosoma*, but may appear singly or as scattered patches in cross-sectional views of the fiber (Fig. 2). In longitudinal sections they appear as elongated tapering areas.

The medulla contains large lightly staining granules held in a vesicular network. There are no fibrils present. The elongated nucleus occupies a more or less central position in the medulla. Frequently the fiber shows a definite enlargement at the region of the nucleus.

Similar fibers have been described for other molluscs. Schwalbe (1869) described a number of gastropod muscles which showed cortical and medullary zones. List (1887) described such muscle fibers in *Tethyls*; Agersborg (1923) in *Melibe*; Roskin (1935) in five species of pteropod molluscs; and Marceau (1937) in a number of the species which he studied.

Wackwitz (1892), describing the muscle fibers of heteropods and pteropods, states that the material in the medullary zone appears sometimes granular, sometimes as a vacuolated network, and that the quantity appears to be related to the activity of the fiber.

Schumann (1911) saw in the retractor muscle of *Gadinia* a lightly staining central core and a fibrillated cortex. He also described a type of cross-striation in occasional fibers. Incidental observations which I made on the radula retractor muscles of *Busycon* indicate that they have a structure identical with that of the radula protractor muscles.

Fibers possessing a fibrillated cortical zone and a clear medulla have been observed in other invertebrates besides the molluscs (Andreae 1881, in *Sipunculus*; Apel 1885 in *Priapululus* and *Holicroptus*; and Jameson 1889 in *Thalassema*).

As in the sheets of vertebrate smooth muscle, connective tissue occurs very sparsely between the individual fibers. Unlike vertebrate smooth muscle, however, connective tissue nuclei are found in the interstices between muscle cells (Fig. 3). The proportion of connective tissue and tissue space is somewhat less than in the retractor muscles of *Phascolosoma* (Olson, 1940) and considerably less than in the retractor muscles of *Thyone* (Olson, 1938).

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SEASONAL GONADAL CHANGES IN THE ADULT OYSTERS, *OSTREA VIRGINICA*, OF LONG ISLAND SOUND

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INTRODUCTION

Gonads of the American oyster, *Ostrea virginica* Gmelin, undergo pronounced changes during the year. Such seasonal changes have been noted in several other Pelecypod mollusks, gonads of which have been studied throughout the annual cycle (Coe, 1932a and 1936a; Orton, 1933; Roughley, 1933; Loosanoff, 1937a; Coe and Turner, 1938). In the ripe adult oyster the gonad is the largest, most conspicuous organ, forming one continuous mass around the folds of the digestive tract and digestive gland, giving the oyster a typical cream-colored appearance. In some years, when gonads are especially well developed, the thickness of the gonadal layer before spawning may reach 1.0 centimeter. During the spawning period, simultaneously with the discharge of gametes, the thickness of the gonadal layer gradually decreases, and in spent oysters the layer becomes extremely thin. Naturally such radical changes, which usually correspond to definite seasons of the year, must markedly affect the physiological behavior of the oyster as well as the chemical composition of its body. Therefore, better knowledge and understanding of the changes occurring in gonads of oysters during different seasons of the year should be helpful in the interpretation of other seasonal phenomena. With this in view a systematic description of seasonal gonadal changes of the adult oysters is offered in this article.

The bulk of material used in these studies was collected from the oyster beds established by the author in Long Island Sound near Milford, Connecticut. These beds were located at different depths ranging from mean low water to 40 feet. Samples were collected over a period of several years at bi-weekly intervals, except in summer when they were taken at least once a week. In all cases the oysters comprising the samples were from four to six years old. Soon after the collection of samples, the oysters were opened and pieces of their gonad tissue placed in a fixing solution, and later prepared for histological studies.

In discussing the changes occurring in the gonads of oysters during

the different seasons of the year, it will be convenient to begin with the discussion of conditions as observed to exist in the winter.

The writer wishes to express his thanks to Mr. James B. Engle for taking the photomicrographs shown in this article.

WINTER OR INACTIVE STAGE

During the cold season, usually beginning with December, the oysters remain in a dormant state. Examination of hibernating oysters shows that during that period their gonads are also in the state of quiescence. The gonad follicles are few in number and they are scattered in the form of small islands throughout the vesicular connective tissue in the area between the body wall and the digestive gland. Most of the follicles are found in that portion of the connective tissue which lies near the genital ducts, the cilia of which remain easily distinguishable during the winter stage (Fig. 1). The follicles are connected with others by fine strands of connective tissue.

The vesicular connective cells are present in large numbers in the area confined between the genital ducts and the digestive gland. These cells are often seen completely surrounding the gonadal follicles and filling the space between them (Fig. 2). In some cases, groups of phagocytes may be noted infiltrating between the vesicular connective tissue cells, approaching the follicles. Such amoeboid cells with darkly stained nuclei are also frequently observed inside the lumina of the follicles where they may be seen grouped around pycnotic cells, apparently performing phagocytic functions.

The follicles of the winter gonad are small in size and almost devoid of lumina. The sex cells usually form but a single row along the follicular wall, the inner layer of which consists of germinal epithelium. Even during the winter stage the sexes are distinguished from each other because of the large number of young ovocytes present in the female gonad. Such ovocytes are easily detected because of their large clean nucleus and granular cytoplasm. These, as well as the indifferent cells, are always prominent in the wintering gonad of a female oyster (Fig. 1).

PLATE I

EXPLANATION OF FIGURES

FIG. 1. Winter gonad of female oyster showing small follicles containing young ovocytes. Part of genital duct is visible. $\times 225$

FIG. 2. Winter gonad of male oyster. Follicles are separated by cells of vesicular connective tissue. Many phagocytes are present. $\times 225$

FIG. 3. Gonad of female oyster showing the beginning of early spring growth. $\times 125$

FIG. 4. Gonad of male oyster in early spring. $\times 125$

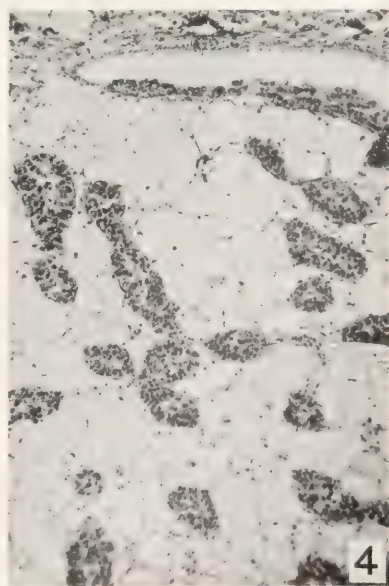
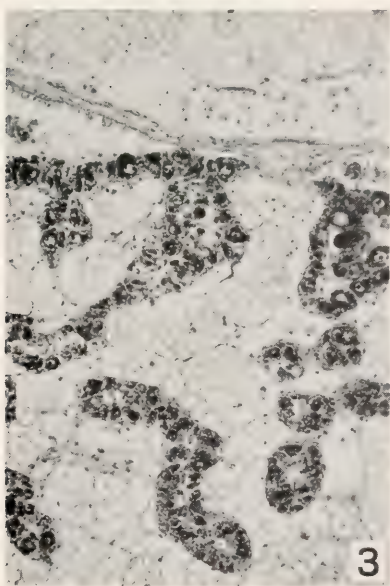
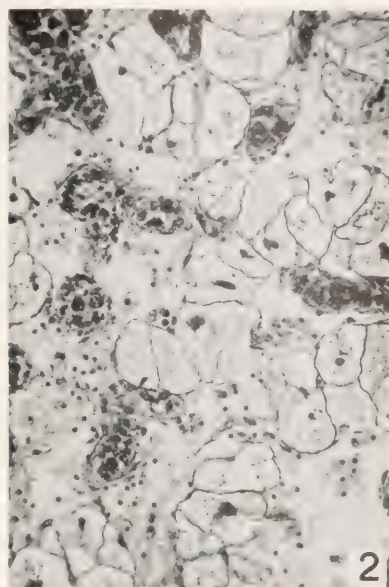
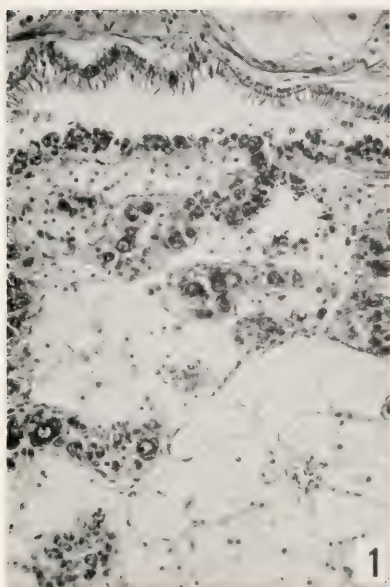


PLATE I

In males (Fig. 2) the follicles are also small and are located throughout the connective tissue in the same way as described for females. They contain indifferent residual cells, spermatogonia and occasionally very few primary spermatocytes. At this, as well as at all other times, when the sex of the oyster can be distinguished, many adult animals of both sexes continue to indicate the unstable character of their gonads. This is especially evident in the males where small ovocytes may be often present along the follicular walls. In the females, groups of cells resembling those of early stages of spermatogenesis may sometimes be detected even in ripe ovaries. Coe (1932*b*) reported similar observations in the case of young oysters, and later (1936*b*) discussed at length the question of the instability of sex in many mollusks.

SPRING DEVELOPMENT AND PRESPAWNING STAGE

Early in April, with the arrival of warmer weather, the gonad follicles in some oysters may begin to show an increase in size, and in the number of cells. Such a growth is usually very insignificant. As a rule, a majority of oysters examined at that time showed that their gonads still were in the state resembling the winter condition. Later in April, however, the follicles of many oysters of both sexes slowly begin to enlarge. In females (Fig. 3) young ovocytes begin to grow extending toward the center of the follicles, and in males (Fig. 4) proliferation and anastomosis of the follicles also become more apparent. They now contain comparatively large numbers of primary and secondary spermatocytes, and in some cases, even spermatids are formed.

In May, soon after the water temperature reaches and passes 10.0° C., very rapid growth and ramification of gonadal follicles commence. They are seen anastomosing through the connective tissue from the region of the genital ducts toward the liver and also in the direction of each other (Figs. 5 and 6). In follicles of the female oysters young ovocytes become larger and more numerous. In males spermatogenesis proceeds at quite a rapid pace, and in many animals spermatids and spermatozoa are already formed in large numbers. In some cases apparently ripe sper-

PLATE II

EXPLANATION OF FIGURES

FIG. 5. Gonad of female oyster in May showing rapid growth and ramification of the follicles. $\times 125$

FIG. 6. Male gonad in May. Ripe spermatozoa are already formed. Cells of the connective tissue are gradually disappearing. $\times 125$

FIG. 7. Gonad of female oyster approaching ripeness. $\times 125$

FIG. 8. Gonad of male oyster of nearly ripe stage. Spermatozoa are very numerous. $\times 125$

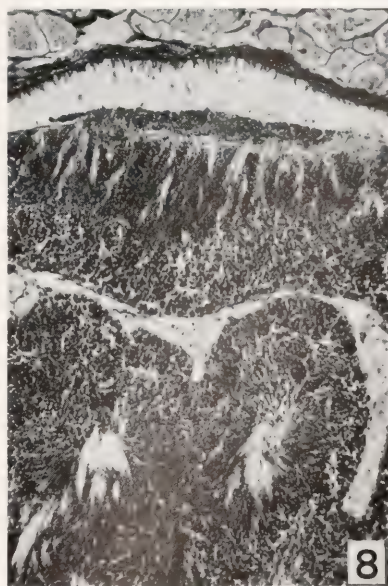
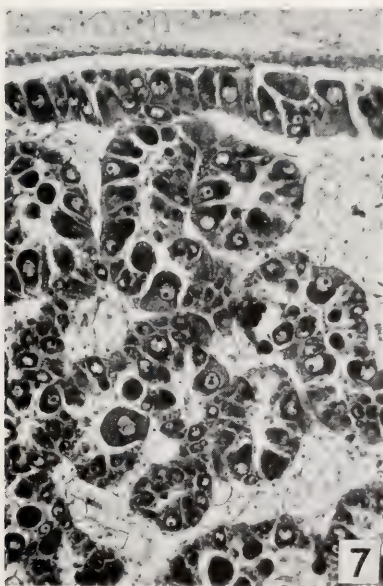
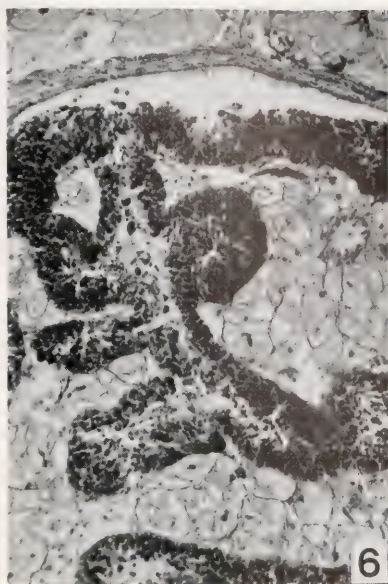
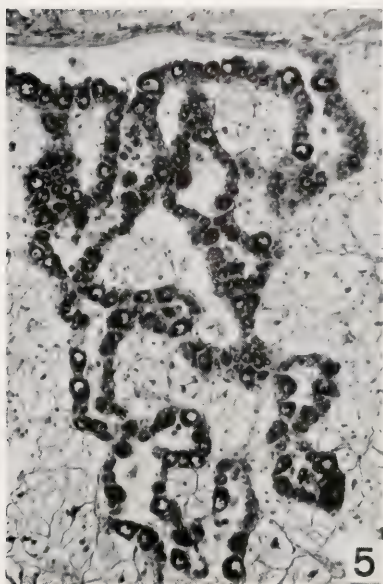


PLATE II

matzoza are noticed entering the genital ducts which are situated beneath the surface epithelium of the oyster body. One of the inner surfaces of the ducts, nearest the body wall, is lined with ciliated epithelium, while the surface facing the gonad is lined with germinal epithelium which gives rise either to male or female sex cells, depending upon the sex of an individual oyster.

In late spring the vesicular connective tissue surrounding the follicles occupies considerably less space than earlier in the season. Roughley (1933) working on the Australian oyster, *O. commercialis*, expressed the opinion that such connective tissue is continually absorbed by the follicles, and is used for the nourishment of the developing sex cells. Coe and Turner (1938) also attribute nutritive functions to such cells. The writer is inclined to accept the same point of view in regard to the connective tissue surrounding the developing gonadal follicles of the adult oysters. As can be seen from figures 3 to 7 inclusive, the connective tissue lies in close contact with the walls of the follicles to which developing sex cells are attached. The nutritive substances contained within the connective cells are probably diffused through the cellular membrane and are assimilated by the sex cells. Such a view merits consideration because in many areas of the gonadal space the connective tissue cells are the only ones surrounding the follicles. The other type of cells, which in some Pelecypoda perform phagocytic-nutritive functions (Loosanoff, 1937*b*), are either absent or found in too small numbers to be considered of importance.

During the first part of June gametogenesis proceeds at a very rapid rate. The gonadal follicles ramify rapidly, and the development of gametes of both sexes is very fast. The vesicular connective tissue, which was so prominent in the spring, begins to disappear (Figs. 7 and 8). In females the ovocytes show pronounced development and some of these cells acquire almost a mature appearance (Fig. 7). In males the sper-

PLATE III

EXPLANATION OF FIGURES

FIG. 9. Section of gonad of ripe female oyster. $\times 125$

FIG. 10. Section of gonad of ripe male oyster. Cells of connective tissue are virtually absent. Mature spermatozoa occupy the follicles. $\times 125$

FIG. 11. Section of gonad of partially spawned female oyster. The follicles are in contracted state. Numerous phagocytic cells are present. Cells of vesicular connective tissue reappearing. $\times 125$

FIG. 12. Gonad of female oyster at the end of spawning. Gonadal layer, confined between the genital duct and digestive gland, is very thin. Phagocytic cells are numerous and active. Gonadal follicles are shrinking. Few eggs are in genital duct on their way to be discharged. Large numbers of connective tissue cells are seen near the section of digestive gland. $\times 225$

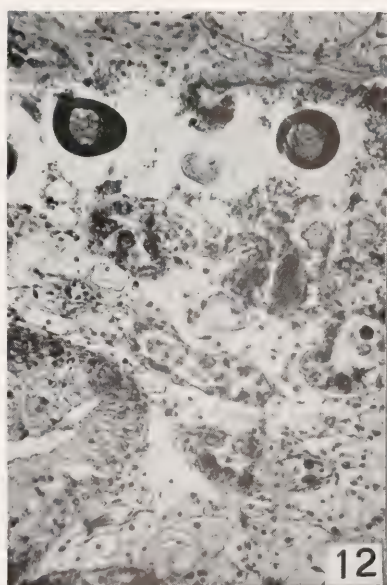
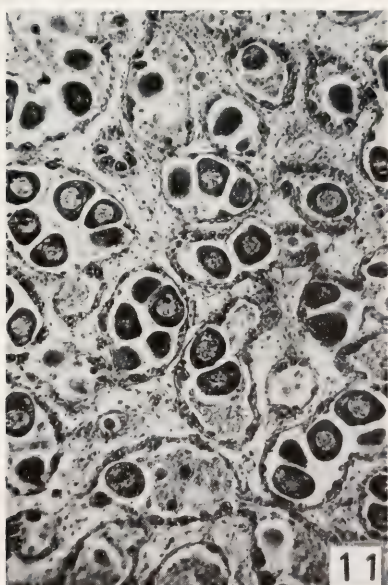
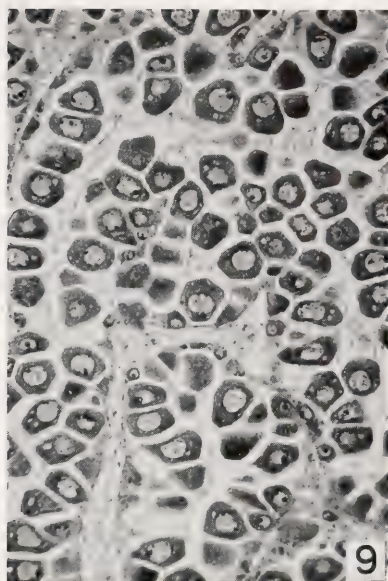


PLATE III

matzoza are more numerous than any other stages of spermatogenesis (Fig. 8).

Toward the end of June, many oysters reach a fully ripe stage. Branches of anastomosing follicles reach the region of the digestive gland, and are separated from it only by a thin layer composed of a few cells of connective tissue. Follicles are now large and distended. In females they contain mainly ripe eggs, although young ovocytes and oögonia are also present along the follicular walls (Fig. 9). The majority of these cells develop later in the season. As a rule, newly formed follicles in the region near the digestive gland contain more cells in the early stages of gametogenesis than the older follicles located nearer the genital ducts. The quantity of the interfollicular vesicular connective tissue diminishes further. Phagocytic cells can be seen outside the follicles, but usually none are found in the lumina.

In males the central part of the follicles is occupied by large numbers of mature spermatozoa (Fig. 10). Masses of them may be seen protruding into the genital ducts, ready for discharge. The cells at the earlier stages of spermatogenesis, which are usually found near the follicular walls, are few in number.

PARTIALLY SPAWNED STAGE

At the proper time, upon receiving certain stimuli among which temperature and chemical impulses are known to be of importance, the oysters begin to discharge their spawn into surrounding water. In Long Island Sound, this usually happens at the end of June or during the first week of July (Loosanoff and Engle, 1940). As a rule, an individual oyster does not discharge its entire content of sex cells at one time. Usually the spawning is extended through many days or even weeks.

The gonads of partially spawned oysters are always easily recognizable. They are characterized by the shrinkage of the follicles from which some of the eggs have already been discharged (Fig. 11). The remaining undischarged eggs are closely grouped together. In males, the central portion of the follicles is often empty because of the recent discharge of large quantities of ripe gametes. Usually almost the entire content of the follicles consists of mature spermatozoa, although a few of the cells of the earlier stages of spermatogenesis can always be found near the follicular walls. In both sexes numerous phagocytic cells appear in interfollicular spaces, and some of them proceed to invade the follicles.

A few cells of the vesicular connective tissue may be seen appearing in the newly formed interfollicular spaces of shrinking gonads.

ADVANCED POST-SPAWNING STAGE

As the spawning progresses and the oysters enter more advanced stages, a still greater shrinkage of follicles occurs (Fig. 12). There are but few undischarged eggs left, and many follicles are entirely free of them. Phagocytic cells appear in very large numbers, both inside and outside the follicles. They are engaged in devouring and cytolyzing remnants of undischarged gametes and pycnotic cells. At this stage resorption of the gonads proceeds very rapidly and soon, emptied follicles begin to acquire an elongated form. Finally, the lumen almost entirely disappears and the walls of the follicles come virtually in contact with each other. The gonadal layer rapidly diminishes in thickness and the distance between the surface epithelium and the digestive gland of the oyster becomes very small. In the majority of cases examined, cytolysis and the resorption of gonads were completed by the end of October.

It was already mentioned that during the ripe stage there are but few cells of the connective tissue found in the interfollicular spaces. Such cells, however, are found, at that time, in large numbers between the tubular folds of the digestive gland. In the advanced spawning stage, following the invasion of phagocytes, the cells of the connective tissue of the digestive gland region begin to proliferate. Soon masses of these cells may be seen penetrating into the interfollicular spaces gradually surrounding the shrinking follicles. The number of these cells increases parallel with the resorption of gonads, and they reach the greatest abundance at the time when the oysters enter into the winter stage.

INDIFFERENT STAGE

As the resorption of the undischarged eggs and spermatozoa is completed, the gonads gradually enter into the indifferent phase. The follicles become very small in size, and are separated from each other by large masses of connective tissue. The follicles are free of all but indifferent sex cells, and as a result it is impossible, at this stage, to determine the sex of an individual. It is thought, therefore, that sex reversal, which is known to occur in American oysters (Burkenroad, 1931; Galtsoff, 1937, and others), takes place during this stage, when sexuality of an oyster is least defined.

SEX DIFFERENTIATION STAGE

After the end of the indifferent period, which usually takes place in late October, slight gametogenic activities are occurring in the oysters. In females numerous ovogonia and small ovocytes are developing along

the follicular wall, while in males a few primary spermatocytes may be found in some follicles. In both sexes the follicles show slight expansion, and animals of different sexes are again easily distinguishable. The fall gametogenesis is, however, of short duration, and as compared with that of the spring, is much less active.

With the arrival of cold weather, the oysters enter into the hibernation period, and their gonads remain in a dormant stage until the next spring.

DISCUSSION

Studies of the changes taking place in the gonads of adult oysters indicate that during the annual cycle these animals experience two periods of gametogenetic activities. The first of these occurs in the autumn, immediately after the indifferent period. It is, however, of very short duration, being interrupted by the onset of low temperature which induces hibernation of the oysters. During the autumn period the cells of only the early stages of gametogenesis are formed. The second period occurs in the spring and early summer and during it the gametogenesis is completed. These observations are in agreement with those of Coe (1932*b*) who reported that in young individuals of *O. virginica* of the Long Island Sound and Woods Hole region, the process of spermatogenesis does not continue through the winter, and no spermatozoa are formed before spring.

The observations made during these studies point to the conclusion that, as first suggested by the author (Loosanoff, 1936) sex reversal, when it occurs, takes place during the indifferent stage when the sexuality of an individual is the least apparent. During this stage, which is of very brief duration, the oyster gonad contains undifferentiated cells only. The further development of these cells, along male or female lines, is probably influenced during the indifferent period by some factors, the nature of which is not very well understood at present. Later in the season, regardless of the fact that gonads still retain their bisexual potencies, the sexes are already strongly defined, and, therefore, it is very improbable that reversal of sex can occur then.

In comparing seasonal gonadal changes of *O. virginica* with those of other species of the oviparous type of the genus *Ostrea*, it will be noted that, in general, they are rather similar. Amemiya (1929) found that in the Japanese oyster, *O. gigas*, the chief gametogenetic activities occur mainly in spring. A similar observation is reported on *O. commercialis* (Roughley, 1933), in which case the gonads are in a resting stage in winter time.

If *O. virginica* is compared with some pelecypods, other than oysters, significant differences may be noted in certain stages of the annual cycle. For example, in the hard shell clam, *V. mercenaria*, contrary to the conditions observed in the oyster, the most active period of gametogenesis and the production of gametes occur in the autumn and early winter. Morphologically ripe spermatozoa and ova are retained by the clam through the winter (Loosanoff, 1937a, 1937b).

Observations presented in this article describe the gonadal changes that occur at certain seasons of the year in the majority of oyster population of New England waters. Often, however, individuals found in the samples may differ greatly in their conditions from the other oysters collected at the same time and place. Such oysters may exhibit either advanced or retarded gonad development. In one case, for example, a large number of oysters collected on March 20, 1939, in Narragansett Bay contained a female oyster whose gonad resembled the partly spawned condition which is usually observed in July. Apparently, spawning of this individual was delayed, and the animal entered the hibernating stage before discharging all of its spawn. It is unusual, however, that in that case there was no evidence of cytolysis or resorption of unspawned eggs, which in all respects appeared normal and healthy.

In another case of similar nature, a male oyster, with gonad filled with spermatozoa, was found in a sample of oysters taken from Long Island Sound in the middle of March, when the water temperature was near 0° C. When some of this gonad material was placed in a drop of sea water and examined under a microscope, the spermatozoa could be seen actively swimming in the surrounding medium. Histological examination showed that the content of the gonadal follicles consisted largely of ripe spermatozoa, whereas the cells of earlier stages of spermatogenesis were very few. This observation indicates that ripe spermatozoa found in the male were the product of the previous year's gametogenetic activities. Therefore, it is quite possible that in some rather rare and unusual cases an oyster may retain large quantities of ripe spermatozoa throughout the winter.

A wide difference in the degree of maturity of oysters taken from the same bed and at the same time has been reported by Nelson (1928) who conducted his observations in Barnegat Bay.

In collecting samples from the beds of Long Island Sound, an opportunity presented itself to compare the condition of the gonads of oysters living at different depths. As far as the beginning of spring gonad development was concerned, very little difference was observed between the groups of oysters brought from the depths ranging from mean low water mark to 40 feet. Although some of the oysters in each group were

in either advanced or retarded stage, the majority of oysters, regardless of the depth they came from, were in about the same stage. It was noted, nevertheless, that prior to the beginning of spawning the oysters of shallow water accumulated much larger quantities of spawn than the deep water oysters. Within the depth range studied the beginning of spawning occurred about the same time. However, the shallow water oysters usually completed spawning somewhat earlier than the animals living in deeper water.

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OBSERVATIONS ON THE DUAL CONTRACTION OF CRUSTACEAN MUSCLE¹

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Crustacean skeletal muscle possesses characteristics of both smooth and striated muscles of vertebrates. Like smooth muscle, its inhibitory mechanism is peripheral rather than central (Richet, 1879). It does not respond so readily to single induction shocks as to repetitive stimulation. Under appropriate stimuli its contractions are sluggish, and maximal tonic contractions can be maintained for long periods without apparent fatigue (Richet, 1879). On the other hand, the muscle is cross-striated; is under voluntary control; and is capable of quick powerful contraction and prompt relaxation. As in vertebrate skeletal muscle, tonus, when present, is probably maintained through impulses passing to the muscle from its nerve (Barnes, 1930). The two types of response present such sharp contrasts that as a result of his studies on *Astacus*, Lucas (1917) concluded that the adductor muscle of the claw possesses two distinct neuro-muscular systems. He arrived at this conclusion mainly because of the break in the strength-duration curve of excitation associated with an abrupt change in the character of the muscular response. These two types of contraction so characteristic of crustacean muscle were the subjects of the present investigation.

EXPERIMENTAL

Experiments were made on the blue crab (*Callinectes sapidus*), the spider crab (*Libinia canaliculata*), and the lobster (*Homarus*) under temperature conditions prevailing at the Woods Hole laboratory during the summer season. A claw-bearing appendage was removed and placed in a clamp. Stimuli were applied either to the nerve placed on non-polarizable electrodes or directly to the muscle by silver-silver chloride electrodes introduced through small openings in the shell. Induction shocks or condenser discharges were used to excite. Contractions of the adductor muscle were recorded either by attaching a lever directly

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to the muscle tendon or to the movable jaw of the claw, the tendon of the adductor being cut.

Single Brief Electrical Currents

Proceeding as above, single shocks were applied at regular intervals and intensity gradually increased from a subliminal value. Intervals were of sufficient length so that cumulative summation of effects of previous stimuli did not occur. Under such conditions, the quick contraction (twitch) always appeared first. As stimulus intensity was increased, the slow type of contraction appeared as a shoulder broadening the curve (Fig. 1*A*). If the time interval between stimuli is shortened,

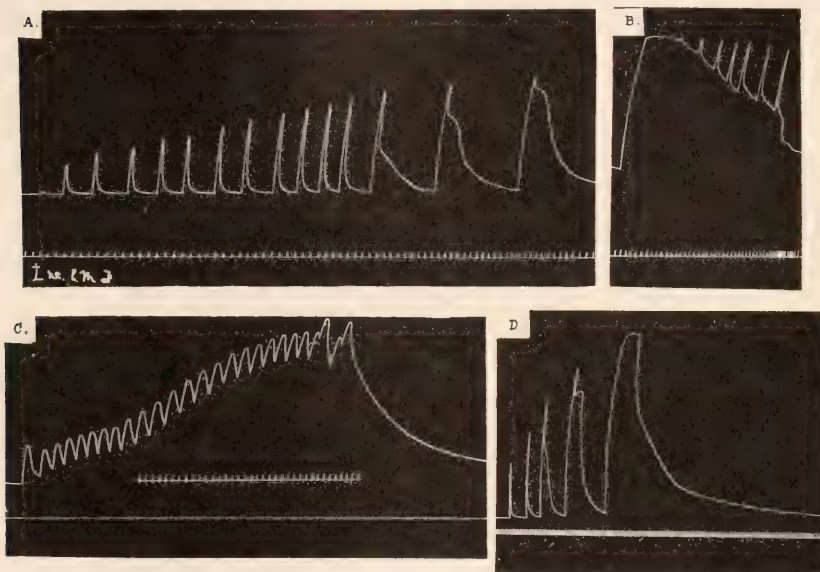


FIG. 1. *A.* Spider crab adductor stimulated directly by single condenser discharges of increasing E.M.F. Time in seconds.

B. Same preparation as *A.* Weaker stimuli applied during relaxation of slow contraction.

C. Spider crab adductor stimulated directly by single condenser discharges. Quick contractions are superimposed on a slowly rising tonus. Time in seconds.

D. Spider crab adductor stimulated through its nerve by single condenser discharges. Fourth and fifth contractions show a slow contraction developing after the quick contraction is over. Time in seconds.

summation effects appear and the quick contractions are added to a slowly rising tonus wave (Fig. 1*C*). When the muscle was stimulated directly by a single condenser discharge or an induction shock, the E.M.F. had

to be increased three or four fold before the slow contraction appeared. With further increase, the contraction became progressively prolonged and frequently lasted several minutes following a single strong stimulus. By a slow repetition of the stimulus, e.g., 1 per second, the contraction under light tension could be continued for more than 10 minutes without evidence of fatigue. During the relaxation of the slow contraction, twitches could be superimposed by applying appropriate weaker stimuli (Fig. 1B). Results were essentially the same whether the stimulus was applied to the nerve or directly to the muscle, except that the nerve rapidly lost excitability under strong stimulation and only responded briefly to the higher intensities used. At the prevailing room temperature (23° C.), the total time of the quick twitch was not especially different from that of amphibian muscle, being in the neighborhood of 0.1 second for *Callinectes* and 0.2 second for *Libinia*. The duration of the slow contraction was a matter of seconds. With strong stimulus, it continued to develop after the quick component was over (Fig. 1D).

Repetitive Stimuli

Repetitive stimulation introduces the possibility that various factors, such as summation, fatigue, refractory or supernormal phases, may modify the result. To give repetitive stimuli, a motor-driven rotating double key was arranged to act as a charge-discharge key for condensers or to deliver make or break induction shocks as desired. The rate of stimulation was controlled by motor speed and reducing gears. By this means stimuli of varying frequency and intensity could be applied as desired. The determination of threshold, by this method, applying repetitive stimulation for brief periods, reversed the results obtained with single shocks. At a frequency below a certain limit any effects observed were those of single stimuli but if an intensity was chosen which was subliminal for the single stimulus and applied repetitively, the slow contractions appeared at the lower rate (Fig. 2C, D) to change to the quick type as frequency or intensity was increased. This change from slow to quick contraction was an abrupt one and not a gradual shortening of the contraction time. The frequency at which the abrupt changes appeared varied with the intensity of the stimulus and the condition of the preparation. This is in agreement with the results obtained on other crustacea by Blashko, Cattell, and Kahn (1931), and by Pantin (1936). Changing the procedure, the intensity of the stimulus was varied at different fixed frequencies. The results were essentially the same as above, i.e., the slow contraction appeared at the lower threshold (Fig. 2E). At very low frequencies a limit was reached where, if intensity was sufficient,

effects were those of single stimuli and quick contractions appeared either singly or as saw-toothed notching on a slowly rising tonus contraction (Fig. 2*A*). With frequencies around 10 per second or higher, the slow contraction always appeared first as intensity was increased from subliminal values and changed to the quick type with further increase of intensity. On slow contractions as they developed or relaxed, quick contractions could be superimposed by application of single stimuli of proper intensity or by brief repetitive stimuli of greater strength or frequency.

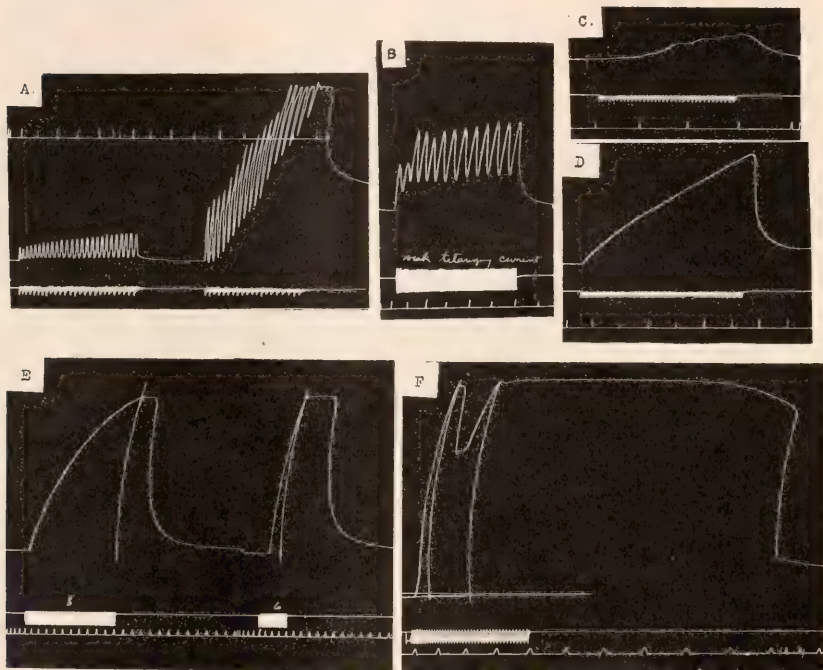


FIG. 2. *A*. Blue crab adductor stimulated by increasing intensity of repetitive induced currents (Harvard induction coil). Signal indicates stimulation frequency (4 to 6 per sec.). Time = sec.

B. Rhythmic quick contractions of lobster adductor stimulated directly by weak tetanizing current (vibrator). Time = sec.

C. Spider crab adductor stimulated at rate of 11 per sec. The slight irregularities are due to variations in output of stimulator. Signal indicates stimulation frequency. Time = sec.

D. Same preparation as *C*. Stimulation rate 14 per sec. Time = sec.

E. Blue crab adductor stimulated repetitively (vibrator) through its nerve with secondary induction of coil at 8 cm. and 6 cm. Lines drawn from crests of the curves indicate differences in time of slow and quick contractions. Time = $\frac{1}{10}$ sec.

F. Blue crab preparation as in *E* shows the development of quick and slow contractions in the same curve. Strong stimulus. Time = $\frac{1}{10}$ sec.

An especially interesting phase of response to repetitive stimulation was the frequent appearance of rhythmic twitches superimposed on the slow contraction. This rhythm when it appeared was quite independent of the frequency of stimulation. These rhythmic contractions appeared at stimulation intensities near the threshold and they often persisted with quite uniform rhythm during the period of stimulation (Fig. 2*B*). It was suggested by Knowlton and Campbell (1929) that they may be due to simultaneous excitation of inhibitory fibers or nerve endings.

DISCUSSION

These experiments show that either the slow or the quick contraction may be excited selectively or both types may be excited simultaneously. When they are excited simultaneously the function of the slow contraction is to maintain the tension developed by the quick contraction. Bronk (1932) has presented evidence that it is the function of the slow contraction to maintain tension with less expenditure of energy associated with a slower rate of nerve discharge. As noted above, this tension can be sustained for a long time without evident fatigue.

With single stimuli of moderately short duration, such as induction shocks or condenser discharges, the quick contraction is elicited at a much lower threshold. With repetitive stimulation it requires greater frequency or intensity. Time relations are not unlike those of the twitch of amphibian muscle. On the other hand, in contrast to these characteristics, the slow contraction with its longer chronaxie or excitation time, has a much higher threshold for single stimuli, except for constant currents of long duration (Lucas, 1917). It is excited by repetitive stimuli of either lower frequency or intensity. With increasing strength of stimulation above the threshold, the contraction becomes more and more prolonged and may persist for some minutes after the stimulus has ceased.

The two types of contraction exhibited by crustacean muscle are so divergent in their characteristics that they suggest that they may be due to different muscle fibers. This was the conclusion arrived at by Lucas (*loc. cit.*). Indeed this possibility has not been entirely excluded. However no corresponding differences in the fibers of a given muscle have been observed, nor have differences in the innervation of the fibers composing any single muscle been described. It seems more probable that each fiber is capable of responding differently as frequency and possibly other characteristics of the excitatory nerve impulses differ. In the evolution of muscle, smooth muscle with its sluggish contraction appears first. With the development of cross striation, ability to rapidly contract and relax appears. More primitive striated muscle like that of crustacea

normally exhibits both types of contraction. In normal skeletal muscles of certain vertebrates such as those of the frog (*Rana temporaria*), the slow contraction (contracture) may still be elicited by appropriate stimuli (Bremer, 1932). Bremer's records of contracture in amphibian muscle find remarkable parallels in the records from crustacea presented in this paper. Such contractures are not the operation of a special contractile mechanism but the expression of a specific type of excitation of the muscle fibers (Bremer). Methods of excitation are generally similar to those causing slow contraction of crustacean muscle. In mammals no contracture has been shown to function in the normal movements of the body though contractures may appear under abnormal and pathological conditions as in certain myopathies or following the section and subsequent degeneration of a motor nerve. These contractures have been reviewed in detail by Gasser (1930). Thus slow contraction is found in all types of striated skeletal muscle. It is a normal mechanism in crustacea but vestigial in mammals. Its specific type of excitation is characterized by the slowness of its development and disappearance. Its long chronaxie requires a single stimulus of long duration (Lucas, 1917) or excessive strength or stimuli of lower intensity or duration applied repetitively so that they produce their effect by summation.

It seems entirely probable that the single stimulus of high intensity acts by setting up a repetitive effect in nerve fibers or nerve terminals. It is not possible to eliminate such effects by curare, since motor endings in crustacea are not paralyzed by curare (author's observation). Verzar and Ludany (1929) observed rhythmic electrical discharges from passage of a constant current through crustacean nerve. Barnes (1930) also noted that a repetitive nerve discharge was set up by strong stimulation as cutting or crushing, and Van Harreveld and Wiersma (1936) recorded repetitive action currents in the nerve associated with the slow contraction. Whatever its nature, the excitation process resulting in the slow contraction is characterized by remarkable powers of summation. Stimulation, even if subliminal, sets up persistent changes which are additive. Contraction may appear only after 40 or 50 separate stimuli extending over a number of seconds. It is this persistence of effects and their consequent summation which explains the selective effectiveness of repetitive stimulation for excitation of the slow contraction. On the basis of evidence at hand, it seems probable that the slow contraction of crustacean muscle can occur only as the result of stimuli which are repeated and summed.

The slow development and disappearance of this additive excitatory effect make it intriguing to consider the possibility that a humoral mechanism is involved. Many of its characteristics, especially its time rela-

tions, could be satisfactorily explained on this basis. Among the known humoral agents of vertebrates, adrenalin causes contraction of crustacean muscle (Du Buy, 1935). Pituitrin (vasopressin) also causes contraction and these contractions are of the slow type (author's observation).

As different frequencies selectively excite different types of contraction, these frequencies could be transmitted by one nerve fiber and it is not necessary to postulate multiple excitatory innervation though this possibility is not excluded. In a series of papers, Van Harreveld and Wiersma (1936-1939) have presented evidence that innervation varies in different crustacea. Examples of single, dual, triple, and even quintuple innervation are described. Even the different muscles of the same individual differ in mode of innervation (Van Harreveld 1939). Multiple innervation furnishing exclusive paths for different activities appears to possess distinct advantages over a single common path though it is not a necessity nor is it uniformly present.

SUMMARY

The slow and quick types of contraction of skeletal muscle of certain crustacea have been investigated and characteristics of the two types of contraction are described. These contractions are correlated with "twitch" and "contracture" of vertebrate muscle.

By appropriate stimuli, one may excite either type of contraction selectively or may excite both simultaneously. When both types are produced by a single strong stimulus, the slow contraction functions to sustain and continue the tension developed by the quick contraction.

Due to the remarkable powers of summation associated with excitation of the slow contraction, it is most easily excited by repetitive stimuli of low intensity. Possibilities of a humoral factor are suggested.

On such a slow contraction, quick contractions may be superimposed by sudden increase of frequency or intensity.

Thus by appropriate stimuli one may excite either type of contraction selectively or may excite both simultaneously.

The function of multiple innervation is discussed briefly.

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WING FORM AND GENE FUNCTION IN NINE GENOTYPES OF *DROSOPHILA MELANOGASTER*¹

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We have reported previously on a number of experiments on the vestigial locus of *D. melanogaster* in which marked changes of wing form were studied (Harnly, 1930*a, b*, 1936, 1940*a, b*, 1941; Harnly and Harnly, 1935, 1936). During the course of those experiments a number of minor wing margin defects were observed at certain temperatures in otherwise constant normal phenotypes. Other genotypes, approaching the wild phenotype in only one part of the viable temperature range, had major or minor wing margin variations in those nearly normal wings. A larval thermolabile period in wing development at high temperatures for the mutant vestigial of *D. melanogaster* has been reported by several workers (Harnly, 1930*b*, 1936; Li, 1936; Stanley, 1931, 1935). The pupal period of vestigial has proved refractory at high temperatures. The following experiments were: (a) an attempt to find a pupal thermolabile period in vestigial; (b) a study of the effects of less extreme alleles and modifiers on wing margin variation; and (c) the determination of the temperature-effective-period of these wing margin variations in preparation for an histological study of wing development during the larval and pupal periods.

TECHNIQUE

Unless otherwise specified, the stocks used in these experiments have all been backcrossed in single pair matings for many generations to males from our inbred vestigial stock (single pair sib matings). These strains should therefore differ only by the mutant genes indicated. Mass matings of the genotypes examined laid eggs for a one or two hour interval on small trays of banana-agar media painted with a heavy yeast suspension. These trays were incubated at 25° C. for 24 hours. Ten recently hatched young larvae were then transferred to each 1 × 4 inch vial containing the yeasted customary banana-one per cent agar medium. This

¹ Aided by a grant from the Penrose Fund of the American Philosophical Society of Philadelphia.

concentration of larvae per unit area is well below the point where crowding affects the wings. The larvae transferred were selected for uniform size. This technique insures uniformity of larval age as any eggs that have been held back by the females after fertilization will have hatched earlier and all these larger larvae were discarded. The high degree of accuracy of temperature control in our incubators has been discussed previously (Harnly, 1936). Vials of larvae transferred from a high to a low temperature (16°) were placed first at 10° for thirty minutes to lower rapidly the temperature of the food mass. The treatment of the larvae is described below for each genotype. The wings of all emerged flies were examined under $15\times$ magnification.

THERMOLABILE PERIOD FOR VESTIGIAL, DIMORPHOS VESTIGIAL, AND WING-MARGIN GENES

One hundred and forty vials of newly hatched vestigial larvae were placed at 32° until they had completed a total of 120 hours of development from the time the eggs were laid. Seventy vials were then transferred to 25° and the other seventy vials to 16° for the completion of development. The wing size and phenotype ("notched") of the flies emerging at 25° and at 16° were the same and similar to those previously reported for transfers at this time interval from 32° to 25° (see Harnly, 1936 for wing drawings and measurements for 32° to 25°).

Vials containing newly hatched vestigial larvae were placed in 32° incubators. Beginning shortly before the onset of puparium formation these vials were examined hourly and recently formed puparia isolated. One-fourth of these were immediately placed at 25° for the completion of development and emergence. The second quarter were placed at 16° for all subsequent development. The third quarter remained at 32° for an additional 24 hours and were then placed at 16° . The fourth group were transferred to 16° where they remained for six days and were then transferred to 25° for emergence. The results in all four groups were identical with each other and with those described above. There was only one significant difference between those transferred at 120 hours and at puparium formation. In the latter animals the wings were much more frequently bilaterally symmetrical in size and general form.

Pupation is extremely difficult at 32° but occurs normally at 28° , 11 hours $\pm$ 30 minutes after puparium formation. Vials containing recently hatched vestigial larvae were placed in 32° incubators. At the time of puparium formation regular inspections were made and puparia formed within the hour were isolated and placed in 28° incubators. The controls remained at 28° for the completion of development and the

emergence of the imagos. After 12 hours at 28° the rest of the vials were returned to 32° where one third remained for 12 hours, one third for 18 hours, and one third for 24 hours. All of these pupae were returned to 28° for the completion of development because homozygous vestigial flies do not emerge at 32°. The results obtained with these three sets of experimental animals were identical with their controls, with those obtained for transfers in this work from 32° to 25° and 16°, and with those previously reported for transfers from 32° to 25° for similar time intervals (Harnly, 1936).

We have reported previously on temperature-effective-period tests of the reciprocal type (Harnly, 1930*b*). In those experiments the development of the vestigial animals began at 29°, one degree below the critical temperature for the increase in wing size (Harnly, 1930*a*). The controls remained at 29° for total development. Groups of experimental animals were transferred at intervals of 24, 48, and 72 hours after egg laying, puparium formation, pupation, and 24 and 48 hours after pupation from 29° to 31° for the completion of development and the emergence of the imagos. Again the thermolabile period ended at the time of puparium formation. From all the experiments reported above it is evident that in the case of the vestigial genotype there is no thermolabile period for the development of wing form during the pupal stage at high, intermediate, or low temperatures.

All of the varied experiments on the vestigial genotype reported above were repeated with the dimorphos vestigial genotype. (Dimorphos is a sex-linked recessive modifier of vestigial.) No temperature response occurred during the pupal period either at high, intermediate, or low temperatures, hence the temperature-effective-period is restricted to the larval stage in this genotype. (For a description of dimorphos vestigial and its general response to temperature see Harnly and Harnly, 1935.)

In previous work with the stock of vestigial-pennant (a reverse mutation from vestigial to a recessive "wild") that had been repeatedly back-crossed to the inbred vestigial strain perfect wild-type wings were produced at 16° on part of the flies; animals developing at all higher temperatures had minor nicks on both wings (Harnly and Harnly, 1936).

Vials of newly hatched larvae were placed at 25° to determine whether this nicking is thermolabile prior to or after puparium formation. Control vials remained at 25° for the completion of development and emergence of the adults. Experimental animals were transferred to 16° at 96 hours after egg laying and at the hour of puparium formation which took place 110–130 hours from egg laying, the bulk of the puparia being formed between 114 and 122 hours. There were no perfect wings on 477 control males and 445 control females nor on 708 males and 552 females

transferred to 16° the hour they had formed puparia. The 96-hour shift to 16° gave 17 per cent of the males with one perfect wing and 3 per cent of them had both wings perfect; 15 per cent of the females had one perfect wing and an additional 6 per cent had both wings perfect. The data demonstrate that the presence or absence of minor nicks in this genotype is determined by the temperature experienced during the larval period prior to puparium formation. Since the frequency of normal wings was not as high as that obtained for total development at 16° the temperature-

TABLE I

Wing characteristics of stock *b vg^p*, reared at temperature indicated, 2 trials.

O—number of flies neither wing showing characteristic indicated, L—left wing shows, R—right wing shows, LR—both wings show

°C.	Wing Variation	Males				Females			
		O	L	R	LR	O	L	R	LR
16	Margin Normal				340				310
25		38	68	69	162	236	47	39	45
30		124	87	88	75	181	42	27	12
30	Margin Normal -V		7	16			16	10	1
25	Extra X-Vein II-III	206	50	49	32	263	51	44	9
30	Extra X-Vein II-III	230	51	59	34	209	16	23	14
	Extra X-Vein III-IV	340	10	23	1	237	14	11	
	Extra X-Vein II-III and III-IV	365	5	3	1	256	2	3	1
16	Delta	258	35	37	8	219	35	41	15

effective-period must begin prior to 96 hours of total development and end after 96 hours but no later than the time the puparia are formed.

WING-MARGIN AND VEIN GENES

It had been observed that the original black vestigial-pennant stock threw a good many males and females with normal wing margins at 25°. Black vestigial-pennant animals were reared at 16°, 25°, and 30°. The data are shown in Table I. They differ markedly from those obtained from the backcrossed vestigial-pennant flies. Both wings of all black vestigial-pennant flies had normal margins at 16° and a fairly large

proportion of the flies had at least one wing with a normal margin at 25° and 30°. The difference in the results with the two vestigial-pennant stocks indicated that the marginal nicks must be due, at least in part, to one or more other loci.

Green and Oliver (1941) have reported "that a larval temperature of 27° is sufficient to increase significantly the wing length of black-vestigial and yellow-vestigial males and females. . . . No such effect was noted among vestigial and vestigial-sooty individuals." The differences between the wings in comparative samples of these genotypes were not only significant but actually profound. We have tested the black vestigial and the yellow vestigial stocks carried in our laboratory at 25°, 27°, 28°, 29°, 30°, 31°, and 32°. All of the males and the females reared at the intervals from 25° through 29° had typical "vestigial" wings. Our previously reported changes in phenotype at 30° and above were also observed in these two stocks. Evidently, these pigment genes do not influence the phenotype of homozygous vestigial and Green and Oliver are dealing with some other modifier of vestigial lowering markedly its critical temperature as does dimorphos (Harnly and Harnly, 1935). Mr. Green informs me that in view of certain internal evidence in his stocks, this interpretation is probably correct.

Four new conditions were observed in the wings of the black vestigial-pennant stock. (1) The distal end of the V-longitudinal vein was absent at 30° in 8-10 per cent of the wings with perfect margins. (2) An extra cross-vein between the II and III-longitudinal veins proximal to and near the anterior cross-vein was present at 25° in at least one wing on 40 per cent of the males and 28 per cent of the females. At 30° this extra cross-vein appeared on the wings of 40 per cent of the males and 20 per cent of the females. (3) An extra cross-vein between the III and IV-longitudinal veins was seen at 30° on 9 per cent of the males and the females; and an occasional animal had both extra cross-veins on the same wing. There was no obvious relation between a perfect or imperfect wing margin and the appearance of these extra cross-veins. (4) A delta appeared at the junction of the posterior cross-vein with the IV-longitudinal vein in at least one wing of approximately 25 per cent of the males and the females at 16°.

TEMPERATURE-EFFECTIVE-PERIODS OF MARGIN NICKS, EXTRA CROSS-VEINS, DELTA, AND INCOMPLETE V-VEIN

Transfers were made from 30° to 16° to determine the period during development when these conditions are thermolabile. The results are

shown in Table II. The data indicate that the temperature-effective-period for marginal nicks (all animals normal at 16°) begins at approximately 60 hours in both sexes and ends for the females at about 90 hours and for the males around 96 hours of total development. Mr. Herbert J. Levin (unpublished) has found that the mean time from egg laying of the molt between the second and the third instar in our inbred vestigial stock is 68 hours at 25° and 59 hours at 31°. Evidently the thermolabile period for marginal nicks in the black vestigial-pennant stock begins with the molt from the second to the third instar and terminates before puparium formation. The temperature-effective-period for both types of extra cross-veins (appear at 30°) must be during the pupal period since these veins were not found in the wings of animals transferred from 30° to 16° earlier than 120 hours. The delta (shows at 16°) appeared with

TABLE II

Stock b vg^p, frequency of flies with normal wing margins, animals developing at 30° are transferred to 16° at the intervals indicated, 2 trials.

Legend O, L, R, LR as in Table I

Wing Variation	Age at Transfer	Males				Females			
		O	L	R	LR	O	L	R	LR
Margin Normal	60 Hours		1		123		3	4	207
	72 Hours		3	3	242	5	26	40	278
	78 Hours		3	5	148	30	47	34	165
	84 Hours	2	8	14	70	64	20	24	66
	90 Hours	13	11	16	14	93	19	19	7
	96 Hours	21	11	11	19	111	19	16	24
	120 Hours	19	36	28	62	136	34	38	20

the usual frequency in the groups of flies from all of these time intervals and would seem to be determined during the pupal period.

The reciprocal transfers from 16° to 30° of individuals isolated at the hour of puparium formation constitutes a critical test of the above interpretations. Both wings had normal margins on all the males and the females transferred from 16° to 30° immediately after puparium formation. Furthermore, some 60 per cent of the males and 54 per cent of the females had the extra cross-vein between the II and III-longitudinal veins on one or both wings. Twenty per cent of the males and 22 per cent of the females had an extra cross-vein between the III and IV-longitudinal veins on one or both wings. Both extra cross-veins were observed in the same wing on one or both wings of 41 per cent of the males and 34 per cent of the females. All possible combinations of these

three conditions were seen on the two wings of different individuals. These reciprocal transfers demonstrate conclusively that the entire temperature-effective-period for the presence or absence of the wing margin nicks is during the third larval instar prior to puparium formation and that the critical period for the extra cross-veins is restricted to the pupal stage. The delta (appears only at 16°) did not show in the wings of any of these animals and therefore has its thermolabile stage restricted to the period following puparium formation. Even with complete development at 30° the frequency of the incomplete V-longitudinal veins is low. Consequently the data from the reciprocal transfers is inconclusive but suggests that the temperature-effective-period for this character follows puparium formation.

BLACK VESTIGIAL-PENNANT/VESTIGIAL (UNRELATED)

The marked difference between the data of the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) and the black vestigial-pennant stocks on wing margin nicks, delta, extra cross-veins, and V-longitudinal vein, indicate that these two stocks of vestigial-pennant must differ by one or more other wing determining genes. Experiments were performed to determine something of the number and nature of these other wing genes.

We have shown previously that the heterozygote between the inbred vestigial stock and the vestigial-pennant stock that had been repeatedly backcrossed to it produced deeply notched wings at 32° and "cut," "antlered," and "strap" wings at lower temperatures (Harnly and Harnly, 1936). The original black vestigial-pennant stock was crossed to a non-inbred vestigial stock. This heterozygote produced rather large numbers of males and females at 32° with both wings having perfect margins. The flies with imperfect wing margins at 32° had only very minor nicks in the distal end of the wings between the III and IV-longitudinal veins. Extra cross-veins appeared fairly frequently in the wings of the flies reared at 32°. At lower temperatures the wings were markedly defective and similar to those reported previously (Harnly and Harnly, 1936). The difference in the wings produced at 32° by these two identical vestigial locus heterozygotes must be due to differences in other loci; one set of modifiers producing deeply "notched" wings, the other perfect wild-type wings.

DOMINANT-RECESSIVE DETERMINATIONS

Reciprocal crosses were made between the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) and the black vestigial-pennant stocks to determine whether the modifiers causing the delta, extra

cross-veins, an incomplete V-longitudinal, and margin nicks are dominant or recessive, sex-linked or autosomal. The offspring were reared at 16°, 25°, and 30°. The results are shown in Table III.

TABLE III

Variation of wing characteristics, F₁ of reciprocal crosses reared at temperature indicated, 2 trials. Legend O, L, R, LR as in Table I

	Wing Variation	°C.	Males				Females			
			O	L	R	LR	O	L	R	LR
b vg ^p ♂ × b ⁺ vg ^p ♀	Margin Normal	16	1	16	13	129	2	18	15	153
		25	116	11	18	3	103	26	16	6
		30	56	72	72	112	93	32	28	205
	Delta	16		3	1			12	6	1
	Extra X-Vein III-IV	30							1	
b ⁺ vg ^p ♂ × b vg ^p ♀	Margin Normal	16	2	7	7	170	2	9	8	186
		25	59	34	42	31	78	33	26	10
		30	5	45	43	396	36	33	31	344
	Delta	16		1	1			9	6	
	Extra X-Vein III-IV	30		3	3				3	

An extra cross-vein between the II- and III-longitudinal veins in one or both wings was found frequently on the black vestigial-pennant males and the females at 25° and 30° (Table I). This extra cross-vein was not observed on any of the offspring from the reciprocal crosses at either 25° or 30°. The extra cross-vein between the II- and III-longitudinal veins is evidently due to an autosomal recessive.

An extra cross-vein between the III- and IV-longitudinal veins was observed on some of the black vestigial-pennant males and females reared at 30° (Table I). The data from the reciprocal crosses seems to indicate that this extra cross-vein is controlled in part by a semi-dominant sex-linked gene carried in the X-chromosome of the black vestigial-pennant stock. However, its expression must be modified by an autosomal gene in the backcrossed vestigial-pennant stock since the frequency in the sons out of black vestigial-pennant by vestigial-pennant is markedly reduced.

The evidence is inconclusive on the mode of inheritance and dominance of the gene occasionally causing an incomplete V-longitudinal vein at 30°.

The delta appears with a lower frequency in both sexes among the offspring of the reciprocal crosses and is probably due to an autosomal semi-dominant not finding expression as frequently in the heterozygote as in the homozygote. In the homozygote it appears significantly more frequently in the females than in the males. In the heterozygote this sexual dimorphism in frequency is profoundly increased.

Clear-cut conclusions cannot be drawn on the mode of inheritance of the margin gene or genes. The data of the males seem to indicate that the X-chromosome of the black vestigial-pennant stock carries a gene tending toward normal margins. This is indicated by the higher percentage of males with normal margins from black vestigial-pennant mothers than the reciprocal males with vestigial-pennant mothers. However, the sisters of the first type also have a higher frequency of normal wings than the reciprocal females. These two sets of females should have identical genotypes. Likewise the change in frequency with temperature is disconcerting. A fair percentage of the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) flies have normal wings at 16° but produce no normal wings at higher temperatures. The black vestigial-pennant stock produces only normal wings at 16° and at higher temperatures produces a steadily decreasing proportion of normal wing margins. The reciprocal heterozygotes produce the lowest percentage of normal margins at 25° with markedly higher percentages of normals at both 16° and 30°. Since the two homozygotes have similar temperature responses, a shift in dominance with temperature in the heterozygote corresponding to that found in vestigial/vestigial-pennant (Harnly and Harnly, 1936) does not seem possible, i.e., this difference cannot be due to a change having taken place in the vestigial-pennant gene in one of these two stocks. Probably more than one locus, other than the vestigial locus, is concerned in the production of marginal nicks. It may be that these genes are the domini genes of Goldschmidt but it would take additional work to determine this point (Goldschmidt, 1937a, b; Goldschmidt and Höner, 1937).

PROOF OTHER LOCI CAUSE DIFFERENCES BETWEEN VESTIGIAL-PENNANT STOCKS

A critical proof that the differences between these two vestigial-pennant stocks (extra cross-veins, degree of margin nicking, delta, etc.) were not due to the vestigial locus would come from a transfer of these differences from one stock to another stock. The black vestigial-pennant line was crossed to an unrelated vestigial strain not used in any of our previous work. From the F₁ hybrids two new stocks were developed.

In the first case black vestigial-pennant/vestigial females were backcrossed to black vestigial-pennant males and through forty generations the hybrid females were backcrossed to the original black vestigial-pennant strain (single pair matings). Homozygous vestigial was then obtained by inbreeding the forty first generation. This new vestigial line should carry the I, III, and IV chromosomes and through cross-overs practically the entire II-chromosome of the black vestigial-pennant stock, differing from it only at the vestigial locus and perhaps a few adjacent loci. In the second case the F_1 hybrid females and their daughters through forty generations were backcrossed to males from the unrelated vestigial stock (single pair matings). Inbreeding the forty first generation produced a new vestigial-pennant line which likewise should have had the genes replaced at all loci save the vestigial locus. These two new stocks were then tested.

Larvae of the new vestigial stock were reared at 32° until puparium formation and then transferred to 30° for the completion of development and emergence. A very high percentage of these flies had an extra cross-vein between the II- and III or III- and IV-longitudinal veins (or both) on one or both wings. The presence or absence of these extra cross-veins is not dependent on the gene at the vestigial locus. They are therefore due to other independent loci. The wing phenotype and size were similar to that reported previously for vestigial reared at 32° (Harnly, 1936). The only significant difference observed in wing shape and size was a tendency toward more nearly perfect wings. An occasional fly had only minor nicks or a small notch in the wing at the distal end between the III- and IV-longitudinal veins. This would indicate that the gene or genes for less nicking (normal margins) in the black vestigial-pennant stock had been transferred to the vestigial stock by the repeated backcrosses. It furthermore emphasizes the fact that the size and form of the wing are determined by temperature, the vestigial gene, and other loci as well; in other words, by the entire genotype *and* the environment. The delta at the junction of the posterior cross-vein with the IV-longitudinal vein reported above appears only in the wings of flies reared at 16° . Due to the small size of the vestigial wings at this temperature, it was impossible to test for the presence of this gene.

The new vestigial-pennant stock was tested for the replacement, by backcrossing, of the assumed genes. The data demonstrate that this substitution had taken place (Table IV). Some of these flies differed from the black vestigial-pennant stock by having defective wing margins at 16° but the percentage of flies with one or two normal wings was double that produced at 16° by the vestigial-pennant stock derived from repeated backcrosses to our inbred vestigial line. No perfect wings were

produced at 25°; but at 30° some wings with normal margins were produced. Here again the data differ from that obtained from the other backcrossed vestigial-pennant strain which produced normal wings only at 16° (Harnly and Harnly, 1936). At all three temperatures the data on the wing margin differ from that of the black vestigial-pennant stock (Table I) from which it had been derived by repeated backcrosses to the unrelated vestigial stock. Furthermore, the V-longitudinal vein was complete in all cases, the delta described for the black vestigial-pennant stock was not produced at 16°, and extra cross-veins did not appear at 25° and 30°. A different delta appeared at the junction of the posterior cross-vein with the V-longitudinal vein in the wings of some 14 per cent of the new type vestigial-pennant flies at 16°.

The data from the new vestigial and vestigial-pennant stocks demonstrate that: (1) the frequency of wings with normal or defective mar-

TABLE IV

Frequency of wings with perfect margins, $vg^P F_{40}$ strain, reared at temperature indicated, 2 trials. Legend O, L, R, LR as in Table I

°C.	Males				Females			
	O	L	R	LR	O	L	R	LR
16	21	37	47	222	22	48	50	319
25	357				418			
30	433	4	4		263	25	28	2

gins; (2) the presence or absence of a delta in the posterior cross-vein, and its location; (3) a complete or incomplete V-longitudinal vein; and (4) the presence or absence of extra cross-veins is due to genes independent from the vestigial locus.

DISCUSSION

In these experiments nine genotypes have been subjected to the rigidly controlled environmental variable, temperature. The characters examined were: (1) wing form variation from "vestigial," "strap," and "antlered" through "notched" and, in some cases, "nicked" to "normal" in four genotypes; (2) wing form normal with and without marginal nicks in six genotypes; (3) the presence or absence of extra cross-veins at two unusual points in the wings of four genotypes; (4) the presence or absence of a delta in the posterior cross-vein at either its junction with the IV- or V-longitudinal vein in three genotypes; and (5) an incomplete V-longitudinal vein in two genotypes. The phenotype of each genotype

varied with the temperature experienced during development. When several genotypes had a common character variation the phenotype found at one temperature was characteristic of a specific genotype and was produced at a different temperature by another genotype. In previous papers (Harnly, 1930*a, b*, 1936, 1940; Harnly and Harnly, 1935, 1936) we have reported on a similar temperature response of genes affecting the wing form, the arrangement of the postscutellar bristles, size and form of the balancers or halters, arrangement of the ommatidia, size and form of the eyes, and the presence or absence of wing margin nicks. It is evident that the gene or even the whole genotype does not solely determine the phenotype of the individual.

The genotype of the individual is fixed at fertilization. However, this does not determine the phenotype of the individual. At this moment the limits within which each character may be expressed are set by the genotype of the particular organism. For example, in the dimorphos vestigial stock the wings will never be smaller than "vestigial" and may be as perfect as "normal," the balancers may be minute vestiges or normal in size and form, the postscutellar bristles may point anteriorly or posteriorly, and the eyes may be "normal" or markedly abnormal. The exact expression within these limits is determined separately for each character by the environment within which each individual develops. For example, in dimorphos vestigial the wings, postscutellar bristle, and the eyes change phenotype at different critical temperatures (Harnly and Harnly, 1935). Among the environmental factors that have been demonstrated to act as determiners within the boundaries set by the genotype are: temperature density of population, type or kind of food media, humidity, etc., for *D. melanogaster*. These and other environmental factors have been found acting in other forms of animals and plants. The subjection of the individual successively to two or more points within the environmental variable during the character's critical stage (thermolabile period) results in a phenotype characteristic of neither environmental point. In the case of vestigial the phenotype is an intermediate, the exact expression depending upon the duration of exposure to the two temperatures in the critical period (Harnly, 1936, Fig. 7). The data presented here, in our earlier papers, and in the work of others demonstrates that the environment is affecting the response or action of the genotype in a critical period of some duration during the development of each character. The limits (extremes) for each character of the individual are fixed at fertilization by the genotype established and the phenotype of each individual is *developed* at a particular point within those limits as the resultant of the interacting forces of genotype and environment during the critical stages of ontogeny.

During the course of these experiments a marked difference was observed between several stocks in the frequency of wings with marginal nicks, and previously undetected wing mutants were seen in the original vestigial-pennant stock when it developed at extreme temperatures. The genes for extra cross-veins, delta, and an incomplete V-longitudinal vein were replaced with their normal alleles by repeated backcrossing of the original vestigial-pennant stock to an unrelated vestigial stock. This replacement also changed markedly the frequencies of "normal" vs. "nicked" wing. The reciprocal repeated backcrossing of the unrelated vestigial stock to the original vestigial-pennant line transferred the genes for extra cross-veins to the vestigial strain and, through the shift in what one may call "wing-margin" genes, changed the degree of defect in the wings of vestigial flies reared at a high temperature. Presumably the gene for the delta was also transferred to the vestigial line but due to the form of the wings of vestigial flies reared at 16° it was impossible to test for the presence of the delta gene. The reciprocal tests demonstrated that the two differently located extra cross-veins, the delta, and the frequency of "normal" vs. "nicked" wing margins are all due to a number of different loci and independent in their expression of the particular allele at the vestigial locus. The data presented here and in previous papers demonstrate that the various isolated vestigial stocks have accumulated different and undetected mutations affecting the wing margin, venation, etc. These effects can appear only when crosses transfer the mutant genes to other stocks having wings of normal form or when the vestigial strain happens to develop at an extreme temperature where the wing is more nearly complete and the new mutants can be expressed. Evolutionarily speaking the situation is analogous to a balanced lethal form in which recessive mutants may collect and never show until a rare cross-over occurs. Due to the action of the vestigial gene only the basal region of the wing is developed. Mutant genes affecting potential regions or structures of the wing, which are not developed by the vestigial flies at usual temperatures, may accumulate in the strain even though not expressed. Then an outcross or a single mutation at the vestigial locus will allow the entire group to be expressed suddenly at one moment in time. A similar situation in nature might explain some of the more abrupt and complex changes in evolution.

The loci used in these experiments fall into one or the other of two groups according to the character affected. (1) The alleles at the vestigial locus in the 2-chromosome, at the dimorphos locus in the 1-chromosome, and at the wing-margin loci all affect the amount and form of the wings present. The various combinations of alleles at the vestigial and dimorphos loci determine whether the degree of imperfection

in the wings shall vary from "vestigial" to "notched," or from "vestigial" to "nicked" or "normal," or from "strap" or "antlered" to "notched," "nicked," or "normal." The minor defects on these genotypes are all terminal defects of notching or nicking in the distal end of the wing. The particular combination of wing-margin alleles and the environment determine the range of variation from none to one or more "nicks" in the margins of wings that are otherwise "normal" or approaching normal. The nicks caused by these alleles may appear at any point in the wing margin. (2) The other loci reported here had no detected effect on the amount and form of the wings present. Their effects were on the venation within the wings. An autosomal recessive determined the presence of an exceptional cross-vein between the II and III-longitudinal veins in certain environments; an extra cross-vein between the III and IV veins was similarly determined by an apparently semi-dominant sex-linked gene; the presence in the proper environment of an exceptional delta in the junction of the posterior cross-vein with the IV-longitudinal vein was caused probably by an autosomal semi-dominant, and a fourth locus was associated with the appearance of an incomplete V-vein.

However, there was no consistent temperature response through the viable range within either the wing form or wing venation group of loci and alleles. The vestigial gene for wing form responds directly to increases in temperature but its allele vestigial-pennant responds inversely through the viable range (Harnly, 1930a; Harnly and Harnly, 1936). The results of the reciprocal crosses between the two vestigial-pennant stocks (Table III) and those on the new vestigial-pennant stock (Table IV) demonstrate that some genes for wing-margin defects find expression at low temperatures while others find expression at high temperatures. The same heterogeneous response was found for the group of loci affecting wing venation. One of the genes associated with the extra cross-veins finds expression at both intermediate and high temperatures while the other one is expressed only at high temperature. The gene for an incomplete V-longitudinal vein is expressed only at a high temperature but the one for delta breaks through only at a low temperature. The nature of the response through the viable temperature range is not a function of the type of character or even of the locus. The nature of the response to temperature is a function of the particular allele.

But the stage in development when the gene is thermolabile is not a function of the particular allele or of the locus. Nor would one expect it to be so if the temperature-effective-period has any relation to ontogeny. All of the genes affecting wing form had a more or less common thermolabile period during the larval stage. The data presented here and previously (Harnly, 1930b, 1936; Li, 1936; Stanley, 1935) demonstrate

that the temperature-effective-period at low, intermediate, or high temperatures for the vestigial gene begins at or shortly following the molt from the second to the third larval instar and ends, depending upon the temperature and the modifiers present, during that instar or at its close when the puparium is formed. Similarly the data reported here for dimorphos vestigial demonstrate that its critical period is restricted to the third larval instar. Unpublished data from combinations of alleles at the vestigial locus also demonstrate that the thermolabile period for wing form never ends later than the time of puparium formation. The data reported here for two different combinations of wing-margin genes (nicks) shows conclusively that the temperature-effective-period for both genotypes is restricted to the third larval instar and ends by the time the puparium is formed. One may conclude that genes affecting wing form have their temperature-effective-periods during the larval stage and that this period is ended with the formation of the puparium. Our accumulated unpublished data on many genotypes demonstrate that wing size, in part or whole, is an entirely different problem from wing form and will be considered elsewhere.

The thermolabile period for the genes affecting wing venation is different from that of the genes associated with wing form. The genes at the two loci for extra cross-veins, the locus for the delta, and the locus for the incomplete V-vein were not affected in their expression by the temperature (low, intermediate, or high) at which the larvae developed. The temperature experienced following the formation of the puparium determined whether the exceptional mutant character or the "normal" expression was developed. Mr. Melvin Green has informed me that the temperature-effective-period of vesiculated (vs. 29c) is also during the pupal period of development (unpublished).

Each of these two groups of loci has its own characteristic thermolabile period. Hence, the temperature-effective-period for a character is not a function of the locus or the allele present. This interval must be a function or characteristic of a definite stage in development when the action of either a gene or its product is thermolabile. It seems that both the inception and maximum duration of this period are determined by or linked to general ontogenetic processes. Work now well along in our laboratory on the development of the dorsal mesothoracic discs of three genotypes shows profound morphological changes taking place in these discs and their wing buds during the third larval instar. In studies on post puparium development of a number of mutants affecting venation, Waddington (1939, 1940) has found that minor variation such as extra cross-veins, incomplete veins, and delta effects are determined or differentiated morphologically during his P_2 stage (18-45 hours) after pu-

parium formation. Both his findings on the morphological determination of the wing veins and our tentative conclusions on the morphological development of the dorsal mesothoracic discs and the wing buds would support the conclusion that the thermolabile period for a particular locus is the time of growth and/or differentiation of the structure affected by the gene. Either a precursor material is formed by the gene at an earlier stage and its subsequent action during ontogeny is thermolabile at a specific period morphologically determinable or the action of the gene modifying the development of the character is direct, immediate, and thermolabile at the moment of growth and differentiation of that character. In either case the genetic thermolabile period is a morphological stage of growth and differentiation of the character. The gene or its products cannot affect the development of a character until that stage is reached by the organism, e.g., molt to third instar for wing form, at which that structure is determined, grows, or differentiates. The developing structure then becomes responsive to the action of that gene or the gene products. Our earlier studies have shown that both the rate and the duration of this response is determined by both the genotype and the environment of the individual. If, due to the nature of the allele and the environment, the action is not self-terminated then it is automatically stopped by the attainment of a definite stage in development, e.g., in some genotypes puparium formation for wing form. During this period either the genes causing the minor modifications in the wing venation have not been functioning or the structure is refractory to them or their products. It is as if there was nothing or not enough there yet on which an effect could be made. Following puparium formation the development and differentiation of the wing has progressed to a stage where the final detailed differentiation of the veins can take place. It is not until this stage in ontogeny that these wing vein genes or their products can influence the structure. The thermolabile period is the experimentally determined stage in the development of a given character when the genotype under examination can cut in on and modify the developing structure. Comparative studies now in progress both within the single genotype and between genotypes of the morphological changes during this period should elucidate the effects of the gene upon the mechanics of wing development.

SUMMARY

1. Nine genotypes, including a number of new mutants, affecting the wing form and the wing venation were found to vary in phenotype between 16° C. and 32° C.

2. The dominant-recessive relationships of these new mutants and the linkage group in some cases was determined.
3. The limits for the future phenotype of each character are demarcated at fertilization by the genotype established. The phenotype of each individual is subsequently developed at a point within these extremes as the resultant of the interacting forces of the genotype and the individual's environment.
4. The nature of the response to temperature (positive or negative) throughout the viable range is a function of the particular allele.
5. All of the genes modifying the wing form were found to be thermolabile during the larval period prior to puparium formation.
6. All of the genes associated with minor modifications in the wing venation had their temperature-effective-period during the pupal stage following puparium formation.
7. The interval of the temperature-effective-period is not a function or characteristic of the allele or the locus but of the stage in development when the action of the gene or its products is thermolabile.
8. The rate of the response and the duration, if not the maximum, are determined by the allele and the environment.
9. The onset and maximum duration of this period are determined by definite developmental stages.
10. Apparently the development of the dorsal mesothoracic disc must progress to a certain point before the wing form loci become effective in further growth and differentiation of this structure and its wing bud. Loci causing minor modifications in the wing venation cannot become operative until much later when the final morphological differentiation of the wing is well advanced.

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SOME EFFECTS OF ANDROGENS ON THE ADULT MALE FUNDULUS

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INTRODUCTION

Concurrent with Matthews (1938, 1939a, 1939b), studies (Burger, 1939, 1940, 1941) have been made on the factors which influence the sexual cycle of the adult male *Fundulus heteroclitus*. These authors have found that warm water accelerates spermatogenesis in sexually inactive fish and that cold water retards the appearance of spermatogenesis and delays the testicular involution that follows the breeding period. Day-length has no effect on the testicular cycle. They have further found that *Fundulus* pituitaries implanted into hypophysectomized or normal males will stimulate spermatogenesis. The present study deals with the effects of two androgens,¹ principally testosterone propionate, on the sexual cycle of the adult male *Fundulus*. The literature contains few references to the effects of androgens upon the sexual cycles of adult fish. Pertinent references will be introduced in their proper context.

MATERIALS AND METHODS

In all experiments freshly captured adult males, 7.5–9.5 gm., were used. In each experiment there was the same number of control and experimental fish, and like numbers of fish of approximately the same size. The controls received the same treatment as the experimentals save that hormone was not administered to the controls. For each experiment the environment was identical for the control and experimental groups. These groups were segregated in salt-water tanks. For Experiments 1, 2, 4 the water temperatures were in the main near 13°–14° C.; for Experiment 3 the water temperature was 17°–19° C. Testes were trimmed and weighed after fixation in Bouin's fluid. The precise treatments for each experiment will be found in the *Results*.

¹ Testosterone propionate (Perandren), and testosterone were kindly supplied by the Ciba Pharmaceutical Products, Inc.

RESULTS

The periods covered by these experiments were from the full breeding state (June and July) to complete testicular involution and the preliminary spermatogonial multiplications for a new cycle (August–November). In *Fundulus* there is no abrupt transition from the breeding to the involuted condition in the testis. The gonads do not suddenly cease to form sperm; even when the testes have involuted to almost minimal size, a few sperm continue to be formed. Instead, there is a gradual decline from July to September in the number of sperm formed. The male ducts which are almost wholly intra-testicular in *Fundulus*, also slowly involute from a condition where the ducts are long and distended with sperm to one where they have degenerated into a mass of stromal tissue in which individual ducts can no longer be recognized.

The yellow coloration of the body, characteristic of the breeding male, likewise slowly disappears. Not until late August do the males completely lose this coloration, which is most obvious on those parts of the body where there is the least melanin pigmentation (ventral surface and fins). In the fairly cold water used in Experiments 1, 2, 4, these regressive changes go more slowly than they do in the warmer water of the natural habitat.

Exp. 1. Between June 27 and August 2, twenty-eight adult males, hypophysectomized on June 23–24, each received intraperitoneally, ap-

PLATE I

EXPLANATION OF FIGURES

FIG. 1. Cross-section of a testis from a breeding male at the time when hypophysectomies were performed. The peripheral zone of spermatogenetic activity (s.z.) is broad. The more central duct system (d.) is well developed. Black areas are sperm.

FIG. 2. Cross-section of a testis from a fish kept in a cold-water laboratory tank 6/23–8/8. The spermatogenetic zone is still well developed, but the duct system shows some involution. The testis is smaller than that of a breeding male. Sections from testes from fish injected with 8 mg. testosterone show the same condition as shown in this figure.

FIG. 3. Cross-section of a testis from a control hypophysectomized fish killed 8/1. The spermatogenetic zone (s.z.) is almost devoid of spermatids. The testis is very much smaller than that shown in Figure 2.

FIG. 4. Cross-section of a testis of a control hypophysectomized fish, killed 8/8. Involution is more advanced than that shown in Fig. 3.

FIG. 5. Cross-section of a testis from a hypophysectomized fish, 8/1, which received intraperitoneally 7 mg. of testosterone propionate in oil. Spermatogenesis is more in evidence than in the controls (cf. Fig. 3). The ducts are also better maintained.

FIG. 6. Cross-section of a testis from a hypophysectomized fish, 8/8, which was injected intraperitoneally with 8 mg. of testosterone propionate. The spermatogenetic zone (s.z.) is less active than on 8/1 (Fig. 5), but the duct system (d.) is still well developed.

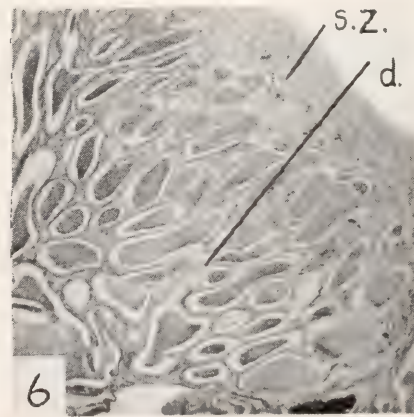
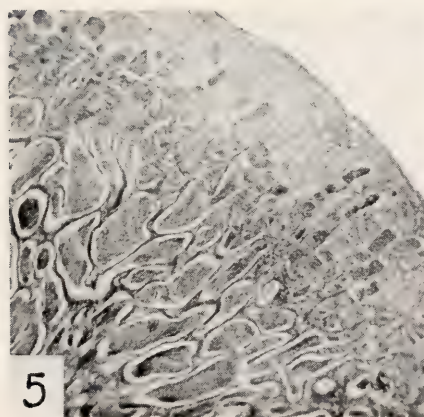
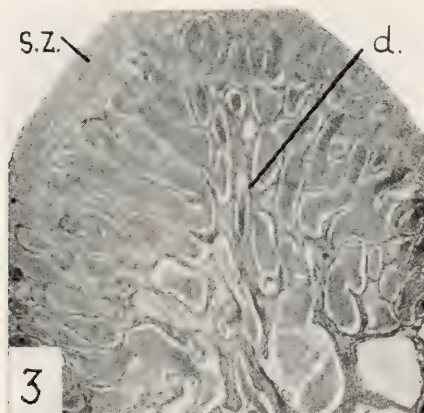
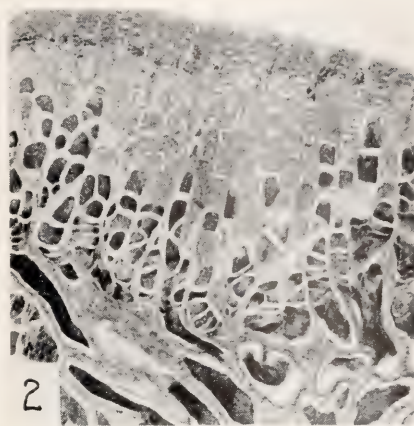
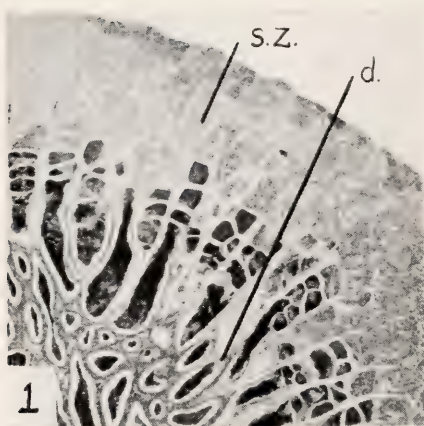


PLATE I

proximately 8 mg. of testosterone propionate dissolved in sesame oil, in equally spaced doses of 1 mg. Fish were sacrificed on August 1 and 8.

At the time of hypophysectomy, the testes were actively forming sperm (Fig. 1); the peripheral zone of spermatogenetic transformations (s.z.) is deep. The more centrally located ducts (d.) are distended and elongate. A section of the testis of a normal laboratory fish at the time of the end of the experiment (Aug. 8) is shown in Figure 2. The only difference between this testis and one from the breeding season (Fig. 1) is a slackening of spermatogenetic activity and a very slight involution of the duct system. The testicular states present in the hypophysectomized controls on August 1 and 8 are seen in Figures 3 and 4. Here spermiogenesis has almost ceased; only scattered cysts of spermatids can be found. The duct system has been markedly reduced. The testes of the hypophysectomized controls were only half as heavy as those of normal controls.

The hypophysectomized fish which were injected with 8 mg. of testosterone propionate over a 42 day period, had testes which weighed² about one-fifth more than those of hypophysectomized controls. Cross sections of the testes of hormonally treated fish are shown in Figures 5 and 6. It is obvious that testosterone propionate has not maintained the testes as well as they were maintained in normal fish (cf. Fig. 2). The involution is not as marked however, as in the hypophysectomized controls (cf. Figs. 3 and 4). Stages of spermatogenesis are more in evidence and the duct system is better preserved. While these differences between hormonally treated and control fish are constant, the testes of the treated fish are also near the end of their spermatogenetic activity. The spermatogenetic-stimulating effects of testosterone propionate with the methods used, are slight. The regressive changes due to hypophysectomy far outweigh any gametogenetic effect of the hormone.

The hormonally treated hypophysectomized fish maintained the full yellow breeding coloration, while most of this coloration was lost in the hypophysectomized controls. Since the nuptial mark (Parker and Brower, 1935) is lost more slowly than is the yellow coloration, a positive statement on this character cannot be made. The contact organs (Newman, 1907) were not studied.

Exp. 2. Between June 27 and August 2, twenty normal males each received intraperitoneally, approximately 8 mg. of testosterone dissolved in oil, in spaced doses of 1 mg. Animals were sacrificed August 1 and 8.

No differences in testicular states were found after a 42 day period between the control and hormonally treated fish. Figure 2 illustrates

² The comparisons of testicular weights must be considered only as rough comparisons.

the condition of these fishes' testes. The hormonally treated fish retained the yellow body coloration better than the controls but less well than it was retained by the hypophysectomized males treated with testosterone propionate.

Exp. 3. Between October 9 and 29, six normal males were injected intraperitoneally with approximately 6 mg. of testosterone propionate in oil in spaced doses of 1.5 mg. Animals were killed November 3.

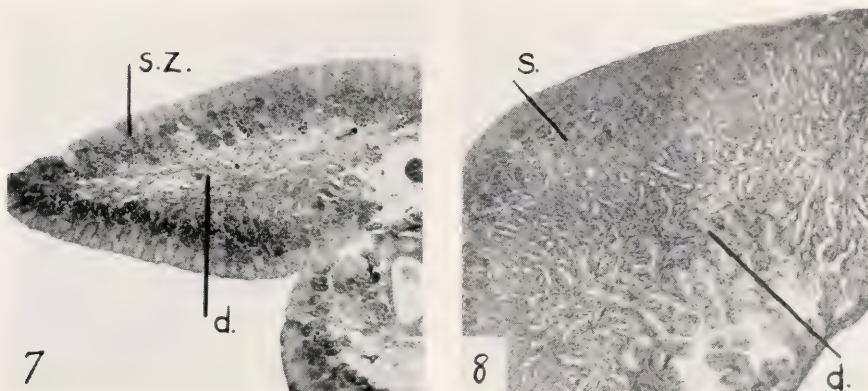


PLATE II

EXPLANATION OF FIGURES

FIG. 7. Cross-section from a normal control male, 11/3, after 25 days in a laboratory tank. The ducts (d.) are represented by the central stromal core, about which is the spermatogonial zone (s.z.) in the early stages of spermatogonial multiplication.

FIG. 8. Cross-section of a testis from a normal fish, 11/3, injected intraperitoneally with 6 mg. of testosterone propionate. The duct system (d.) has hypertrophied and individual ducts are visible. The spermatogenic zone shows spermatids (s.). Essentially the same testicular state was present in fish receiving 3 mg. of testosterone propionate intramuscularly.

On October 9 and 25, six normal males each had approximately 3 mg. (2 doses of 1.5 mg.) of crystalline testosterone propionate implanted into the dorsal musculature. To effect this implantation, crystals of the hormone were tamped into the tip of a hollow glass needle. A wire plunger, snugly fitting the bore of the needle, forced the hormone into the muscles. One scale was removed from the fish. At the site of removal the needle was inserted. The scale was replaced to cover the wound. Animals were sacrificed on November 3.

A section of a normal control's testis is shown in Figure 7. Such a fish was kept 25 days in a laboratory tank. The testicular involution of the previously active gonad has been accomplished, and the prolifera-

tion of spermatogonia for a new cycle has started. Spermatids are as yet rare. The testes of the fish treated with 6 mg. of intraperitoneally injected hormone were about four times as heavy as those of the controls. The testes of the fish in which 3 mg. of testosterone propionate were implanted intramuscularly were about twice as heavy as the controls.

The histological picture was essentially the same for both groups of treated fish, with the testes from the fish injected intraperitoneally being the more advanced. Figure 8 shows a section from a testis from a fish which received intraperitoneal injections of the hormone. The most conspicuous effect of the hormone was the re-elaboration of the duct system of the gonad. Instead of being represented as in the controls by a mass of stroma (Fig. 7, d.), the individual ducts have become individually discernible. The walls of the ducts are thickened and ciliated. The duct tissue now forms the bulk of the testis and is responsible for most of the increased weight of the gonad.

The effect of the hormone on spermatogenesis was again present, but again not marked. In the treated fish there was a slight acceleration of spermatogenesis. This acceleration was not enough to present widely differing pictures between the germ cells of the controls and the hormonally treated fish. While spermatids were much more numerous in the treated fish than in the controls, numerous spermatozoa were not found. It was shown (Burger, 1941) that pituitary material could induce numerous spermatozoa in colder water and in a shorter length of time than the 25 days of this experiment.

The effect of the hormone on the yellow coloration of the body was clear. Within ten days after the first injection, yellow had developed on the body and the fins. At the end of the experiment the hormonally treated fish were brilliantly yellow. In the same period, the controls remained devoid of this color, save for one fish which showed the faintest tint. It must be remembered that the controls were slowly undergoing sexual stimulation from the warm water of the experiment.

Exp. 4. Beginning August 16, ten normal males each received intraperitoneally, approximately 9 mg. of testosterone propionate in oil in 3 mg. doses spaced at intervals of seven days. Fish were sacrificed on September 3.

On August 15, six normal males had approximately 1.5 mg. of testosterone propionate implanted into the dorsal musculature. These animals were killed on September 3.

The results of these experiments were essentially similar to those of Experiment 3. After 18 days of intraperitoneal treatment with hormone the testes were half again as heavy as those of the controls. The fish treated with intramuscular hormone had testes two-fifths heavier

than those of the controls. The hormonally treated fish were more yellow than the controls.

DISCUSSION

In these experiments effects of testosterone propionate were observed upon the yellow coloration of the body, the intra-testicular duct system, and the germ cells. This hormone maintained the full yellow coloration of the breeding male for 42 days after hypophysectomy. In the hypophysectomized controls this coloration was largely lost. In normal sexually inactive males, testosterone propionate, injected either intraperitoneally or intramuscularly, caused a re-appearance of this yellow coloration. Controls did not become similarly colored. In normal breeding males, testosterone, milligram for milligram, was less effective than was testosterone propionate in hypophysectomized males. The results indicate that the suffusion of the body with yellow during the breeding period is normally caused in the adult male *Fundulus* by an androgenic substance. Female *Fundulus* do not show this coloration.

Within the testis the most conspicuous effect of testosterone propionate was upon the duct system. In normal sexually inactive fish the hormone produced a marked hyperplasia of the ducts. In hypophysectomized males, the hormone, with the methods used, only partially maintained the duct system. The duct system was, however, better maintained than in the controls. Testosterone with the methods used had no discernible effect on the duct system.

The effects of testosterone propionate on the germ cells are not unequivocal. In the mammal it has been shown that large doses of androgens maintain the normal condition of the testis if the injections are given before degenerative damage has occurred (Walsh, Cuyler and McCullagh, 1934; Nelson and Gallagher, 1936; Hamilton, 1936; Cutuly, McCullagh and Cutuly, 1937). In the normal adult *Lebistes*, Eversole (1939, 1941) reports somewhat contradictory results. In his 1939 paper testosterone propionate was found to have no effects in experiments of short duration and to be inhibitory to spermatogenesis in experiments of three months duration. In his 1941 paper the hormone is said to have the effects of pregneninolene, which latter substance hastens germ cell maturations without replacements by new cells and causes a hyperplasia of the testicular stroma. Eversole considers the male hormone of *Lebistes* to be more similar to pregneninolene than to testosterone propionate. In the lizard, *Sceloporus*, Forbes (1941) finds that implanted pellets of testosterone propionate stimulate spermatogenesis and produce a hypertrophy of the epididymis and vas deferens. He criticizes the

negative results of Gorbman (1939) as being the result of too short an experiment.

Our results indicate that testosterone propionate has, within the methods used, only a very slight stimulating effect on the male germ cells of *Fundulus*. This effect did not suppress the involution changes that follow hypophysectomy, although the hormone did very slightly retard this involution. In normal sexually inactive fish, this hormone did not markedly accelerate a new spermatogenesis. We have shown (Burger, 1941) that within the time limits of these experiments, pituitary material will produce copious sperm in normal and hypophysectomized *Fundulus*. It is interesting to note that the response to implanted whole pituitaries is primarily one of germ cell activation, and not one of stimulation of the ducts. With testosterone propionate, the effect seems to be the reverse, i.e., marked activation of the duct system and weak activation of the germ cells. The effect noted on the germ cells may not be a direct effect at all. It may be that by stimulating the duct system, which is intra-testicular, the *milieu* of the testis is more favorable for spermatogenesis. It must be remembered that in these experiments the germ cells of the treated fish were not in stages which differed widely from the controls.

It next must be asked whether or not the methods and the dosages employed were adequate for securing pertinent results. In comparison with other data from other workers the use of at least 1 mg. of testosterone propionate per 7.5–9.5 gm. fish per week seems to be a fairly high dosage. The use of intraperitoneal injections of the hormone in an oil vehicle may be questioned on the grounds that it does not provide for a satisfactory absorption of the hormone. Deansley and Parkes (1937) report that intraperitoneal testosterone has little effect in the mammal. Rubinstein and Kurland (1941) do not agree with this conclusion, but Greene and Burrill (1941) cast doubt on the validity of the comparisons made by Rubinstein and Kurland. Experiment 3 clearly shows that in *Fundulus* 6 mg. of intraperitoneal testosterone is somewhat more effective than 3 mg. of intramuscular hormone. The hormone in both methods of administration affected in like fashion the testis and the body coloration. Thus, it can be stated that effective amounts of testosterone propionate can be absorbed after intraperitoneal and intramuscular administration. It is clear from Experiment 2, that the amount of testosterone used was inadequate to produce testicular stimulation.

The intraperitoneal method of administration of the hormone is for small fish more convenient than are intramuscular and subcutaneous methods. In these latter two methods the repeated injection of large

doses is not practical, due to the wounds inflicted and the delicateness of the dermal epithelium. Since *Fundulus* is a very wasteful feeder with non-living food, the oral administration of the hormone would be wasteful of the hormone, and would make a reasonably accurate determination of the dosage difficult.

SUMMARY

Adult male *Fundulus* hypophysectomized during the breeding season were injected intraperitoneally with 8 mg. of testosterone propionate during a 42 day period. The hormone maintained the yellow body coloration characteristic of a breeding male. The hormone did not maintain the testis in the breeding condition. The testicular ducts were maintained better than they were in the controls. There was slightly more spermatogenetic activity than in the controls. Like amounts of testosterone injected intraperitoneally into normal fish over a similar period had no effect on the testis and only partially maintained the yellow coloration of the body.

Normal adult male fish injected intraperitoneally or intramuscularly with testosterone propionate (see text for dosage) during testicular involution and during the early stages of spermatogonial multiplications showed a marked activation of the testicular ducts and a slight stimulation of spermatogenesis. The yellow coloration of the body developed brilliantly in the treated fish. The above experiment was performed once for an 18 day period, and once for a 25 day period.

It is concluded that the yellow coloration of the body, characteristic of the breeding male, is stimulated by the male hormone, and the elaboration of the testicular duct system is influenced by this hormone. While a slight stimulation of spermatogenesis was observed, the degree of stimulation does not warrant the conclusion that in the intact animal the male hormone has any important spermatokinetic role.

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FACTORS INFLUENCING REGENERATION AND POLARITY DETERMINATION IN TUBULARIA CROCEA

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The regeneration of hydroids has been studied extensively, and the subject has recently been reviewed by Barth (1940*b*). Of the environmental factors which effect regeneration in the hydroids, the role of oxygen has been studied in the most detail (Miller, 1937; Barth, 1938*a*; Zwillig, 1939; Rose and Rose, 1941). Several evidences have been presented, however, which indicate that the accumulation of waste products may have an inhibitory effect on regeneration. When the cut ends of *Tubularia* stem segments are covered with glass capillaries, regeneration is inhibited at the covered ends (Barth, 1938*a*). Rose and Rose (1941) showed that accumulated waste products may inhibit regeneration at a cut surface of *Tubularia*, even when a plentiful supply of oxygen is available at the cut surface. Komori (1933) reported that lowered pH may inhibit regeneration. Miller (1939) reversed the normal polarity of *Tubularia* stems by treating the distal ends with a 90 per cent O₂: 10 per cent CO₂ mixture, while the proximal ends were exposed to oxygen.

Attempts to analyze the role of environmental factors in regeneration and the origin of polarity during regeneration are complicated by the heterogeneous nature of the *Tubularia* stem. In the *Tubularia* stem there is a gradient in the rate of regeneration (Barth, 1938*b*), as well as a gradient of oxygen consumption (Barth, 1940*a*). Further complication is introduced by the dominance which distal levels of the stem exert over proximal levels (Barth, 1938*b*). In order to obtain a clear analysis of the role of the environment in regeneration, it is advantageous to work with a homogeneous system. Such a system is provided by the *Tubularia* coenosarc fragments described by Goldin and Barth (1941). When the coenosarc is removed from the perisarc, a series of reorganizational changes occurs involving both morphological and physiological dedifferentiation. A spherical mass of coenosarc cells is formed, in which there is a complete loss of the original polarity of the stem. The polarity relationships which are developed during the regeneration of the coenosarc fragment are therefore new, and have no relation to the original polarity present in the intact stem.

The experiments, using both coenosarc fragments and stem segments, were designed in order to study the effects of oxygen and accumulated waste products in regeneration. They may be described under the following headings: (1) inhibition of regeneration inside of glass caps; (2) determination of polarity by the differential exposure of coenosarc; (3) the extent of acid metabolite accumulation; and (4) the effect of increased hydrogen ion concentration on regeneration. The author wishes to thank Professor L. G. Barth, under whose supervision this work was carried out, and Professor H. B. Steinbach for his helpful suggestions in the preparation of the manuscript.

METHODS EMPLOYED IN THE CAPILLARY EXPERIMENTS

The method of treatment of the coenosarc fragments was essentially the same as that described by Goldin and Barth (1941). Segments of stem ten millimeters long were cut from the region of *Tubularia* stems extending from five to fifteen millimeters proximal to the hydranth. The coenosarc tissues were expressed from the perisarc and were kept on agar (2 per cent agar in sea water) in running filtered sea water, until ready to be used in the experiments. When stem segments were used, they were taken from the same region of the stem (5–15 mm. proximal to the hydranth), but the perisarc was not removed. The capillaries were drawn from clean glass tubing (7 mm. inside diameter) to a diameter of approximately one millimeter. The capillaries were cut into 25 mm. lengths, and when ready for use, were filled with filtered sea water. A capillary was chosen for each coenosarc fragment so that the fragment would fit tightly enough not to be dislodged during the course of the experiment. In order to manipulate the coenosarc fragments without injury to them hair loops were employed. After the coenosarc fragments were inserted into the capillaries, the latter were submerged in running filtered sea water for the duration of the experiment. The capillaries were all kept one half inch below the surface of the water in order to insure uniformity of conditions. The temperature of the sea water during the experiments was $18 \pm 2^\circ \text{C}$. The operations and observations were made with the aid of a binocular microscope.

INHIBITION OF REGENERATION INSIDE OF GLASS CAPS

Barth (1938a) found that regeneration was inhibited when the cut end of a *Tubularia* stem was covered with a glass capillary. This experiment was performed in order to determine whether inhibition could likewise be effected using coenosarc fragments. Dedifferentiated coenosarc

fragments (twenty-four hours after removal from the perisarc) were inserted in capillaries, midway between the two open ends. Stem segments ten millimeters long were placed in the center of similar capillaries. Other coenosarc fragments and stem segments were kept in running filtered sea water as controls.

Regeneration was inhibited in all cases when the coenosarc fragments and stem segments were covered with capillary tubing. Thirty-one coenosarc fragments failed to regenerate one hundred and fifty hours after being placed in the capillaries (174 hours after removal of the coenosarc from the perisarc). Twenty-three out of twenty-five coenosarc fragment controls regenerated ninety hours after removal of the coenosarc from the perisarc. Ten stem segments placed in capillaries showed no evidences of regeneration after one hundred and forty-two hours, while all of the twenty control stem segments regenerated after seventy hours. When coenosarc fragments and stem segments were removed from the capillaries, many of them regenerated, indicating that the inhibition is reversible.

DETERMINATION OF POLARITY BY THE DIFFERENTIAL EXPOSURE OF COENOSARC

Since the covering of a coenosarc fragment by capillary glass tubing is sufficient to cause inhibition, a differential exposure could be accomplished by placing a coenosarc fragment inside the tip of a capillary so that half of the surface is covered by the capillary while the other half is exposed to the sea water in the dish. The coenosarc fragments were placed in the capillaries at time intervals ranging from twenty-four to forty-eight hours after removal from the perisarc. Coenosarc fragments and stem segments were kept in running filtered sea water as controls.

The results of these experiments are summarized in Table I. Seventy-nine coenosarc fragments placed in the tips of the capillaries regenerated new hydranths at the exposed surface of the fragment, while none regenerated at the capped surface. Thus, all regenerants were unipolar, and polarized in the direction of the open end of the capillary. The control coenosarc fragments, on the other hand, gave rise to unipolar, bipolar, bipolar-unipolar, and multipolar regenerants, as described by Goldin and Barth (1941). Since a coenosarc fragment, at twenty-four hours after removal from the perisarc has lost its original polarity relationships (Goldin and Barth, 1941), the polarity of the regenerant is new, and is developed in response to the imposed environmental differential. It may be noted that, although at 48 hours after removal from the perisarc, coenosarc fragments have begun to redifferentiate (Goldin and

Barth, 1941), nevertheless, the differential exposure still determines the polarity of these regenerants. It is indicated, therefore, that the redifferentiation has not yet produced an irreversible determination of polarity.

The factors involved in the differential exposure appear to be oxygen and metabolic waste products. The diffusion rate of oxygen to the coenosarc tissue and of waste products away from the tissue is presumably lower inside the capillary than at the open end. The extent of this effect is indicated by the experiment of Barth (1938a), where inhibition was obtained even when the cut end of the *Tubularia* stem was flush with the end of the capillary. On this basis the coenosarc tissue is subjected to two gradients. There is a gradient of oxygen tension, with the high end at the exposed surface of the tissue, and a gradient of concentration of metabolic wastes, with the high end at the covered surface of the tissue.

TABLE I

The polarity developed by coenosarc fragments placed in the tips of glass capillaries T_1 and T_2 = time in hours after perisarc removal. T_1 = time at which the fragments were placed in the capillaries, T_2 = time at which the observations were made.

Description			Polarity of the regenerants			
Exp.	No. of fragments	T_1	T_2	Regeneration at exposed surface	No regeneration	Dead
1	43	24	96	28	7	8
2	20	39	135	7	2	11
3	18	43	88	4	8	6
4	16	48	163	10	3	3
5	48	48	192	30	12	6
Total	145			79	32	34

Either one, or a combination of both of these gradients might determine the new polarity relationships which develop during regeneration. If an oxygen gradient is the sole factor acting in the determination of the new polarity, it should be possible to obtain regeneration at any point on the coenosarc tissue by the application of a high oxygen tension at any localized point on the tissue, irrespective of the accumulation of waste products. Three experiments were designed in order to test the efficacy of oxygen in the presence of accumulated metabolic wastes.

In the first experiment dedifferentiated coenosarc fragments (26 hours after removal from the perisarc) were placed in the tips of capillaries so that half of the tissue surface was exposed to the sea water in the dish, while the other half was covered with the glass capillary. An oxygen bubble was then introduced into the capillary by means of a micropipette,

at a distance of from two to four millimeters from the coenosarc fragment. The size of the oxygen bubble was such as almost to fill the capillary.

The observations were made 96 hours after the coenosarc fragments were placed in the tips of the capillaries. Out of a total of 37 fragments treated in this way, 14 regenerated at the free end of the capillary, two regenerated in the direction of the oxygen bubble, 11 failed to regenerate, and ten died. Control coenosarc fragments, placed in the tips of the capillaries as in the previous experiment, with no oxygen bubble introduced into the capillary, all regenerated towards the free end of the capillary.

In the second experiment, dedifferentiated coenosarc fragments were placed in the center of glass capillaries containing sea water. An oxygen bubble was then introduced into one end of the capillary, and a nitrogen bubble into the other end of the capillary. The oxygen and nitrogen bubbles were of approximately the same length (8 mm.) and were placed about 2 mm. from the coenosarc fragment. Twenty coenosarc fragments (30 hours after removal from the perisarc) were treated in this way. After 120 hours none of the coenosarc fragments had regenerated. Seven out of ten untreated control coenosarc fragments, free in sea water, regenerated during the same period. It may be noted that the gas bubbles introduced into capillaries showed no appreciable loss in volume during the experiment.

In the final experiment, the coenosarc was removed from the perisarc of the distal half of ten millimeter stem segments, the empty perisarc being left intact. An oxygen bubble was then introduced into the empty half of the perisarc, and the stems were ligated at both cut ends. Untreated stem segments, as well as stem segments from which the coenosarc of the distal half was removed, but in which the ends were ligated without the introduction of an oxygen bubble, were kept as controls. All of the stems were kept in running filtered sea water at $19 \pm 1^\circ \text{C}$.

A total of six out of 60 experimentals regenerated hydranths after 96 hours, in the direction of the oxygen bubble. The oxygen bubbles themselves were reduced, but in no case completely dissipated during the experiment. None of the ligatured controls regenerated during the same period; whereas 29 out of 40 cut, but unligatured, stems regenerated. The experimentals which did regenerate apparently did so because of the increased availability of oxygen. Failure of ninety per cent of the stems to regenerate, despite the presence of the oxygen bubble, was probably due to the inhibition caused by the accumulation of waste products.

THE EXTENT OF ACID METABOLITE ACCUMULATION

This experiment was designed in order to determine the extent to which acid metabolites accumulate when regeneration is inhibited by capping. This could be accomplished by measuring the change in the pH of the sea water inside of the glass tubing. Clean seven millimeter glass tubing was cut into 24 millimeter lengths, and submerged in filtered sea water. Both stem segments and coenosarc fragments were used, one segment or one fragment being inserted into each tube. The ends of the tubing were then plugged with absorbent cotton in order to prevent the stem segments and coenosarc fragments from falling out. Measurements of pH were made with a Beckman pH meter, the water contained in five glass tubes being pooled for each reading. Coenosarc fragments and stem segments were kept in standing sea water as controls.

Twenty-five coenosarc fragments, and twenty-two stem segments were placed in the glass tubes. As in the capillaries, there was complete inhibition of regeneration. The average pH of the sea water in the glass tubes containing coenosarc fragments dropped from 7.96 to 7.11 after 96 hours. With stem segments, the average pH was lowered from 7.96 to 6.90 during the same time interval. Twenty-one out of 35 control fragments regenerated, the pH of the sea water changing from 7.96 to 7.86, while 15 out of 20 control stem segments regenerated with a pH change from 7.96 to 7.90. Thus, associated with the inhibition, there is a lowering of the pH of the sea water.

THE EFFECT OF INCREASED HYDROGEN ION CONCENTRATION
ON REGENERATION

The results of the previous experiments indicated that the accumulation of acid metabolites has an inhibitory effect on regeneration. If the inhibitory action of acid metabolites is due to the increased hydrogen ion concentration of the sea water, it should be possible to obtain inhibition by artificial acidification of the sea water. For an accurate analysis it is necessary to maintain the oxygen tension of the sea water samples at a constant level, while the acidity is varied. The general procedure for adjusting the sea water samples to the desired pH values was essentially the same as that used by Smith and Clowes (1924), and Whitaker (1937), the pH being regulated with acid, base, and buffer. An attempt was made to get a range of pH values from that of normal sea water (pH 8-8.2) down to pH 3. After the addition of acid, the sea water was aerated vigorously in order to re-equilibrate with the atmosphere. This was necessary, since the addition of acid liberates free carbon dioxide.

The pH measurements were made, as before, with a Beckman pH meter, and the dissolved oxygen was determined using the Winkler technique. Two series of experiments were performed.

In the first series of experiments the sea water was acidified to pH 3 by the addition of concentrated HCl. Air was then bubbled vigorously through the sea water for six hours, after which time successive measurements gave constant pH readings. Measured quantities of sea water (100 cc.) were then adjusted to a series of pH values by the addition of the following reagents: 0.2M NaOH; 0.6M NaHCO_3 ; 0.2M NaOH—0.6M NaHCO_3 ; and McIlvaine's buffer (Citric acid 0.1M—secondary sodium phosphate 0.2M). The sea water made up to the different pH values was poured into two-inch finger bowls placed in a constant temperature bath at $19 \pm 1^\circ \text{C}$. Stem segments taken from the region 5–15 mm. proximal to the hydranth were placed in each finger bowl and samples of sea water removed for determination of pH and dissolved oxygen. The stems were observed for regeneration when, by gross inspection, most of the control stems in normal sea water had formed primordia. The pH and the oxygen tension were determined again at the close of the experiment.

In a second series of experiments the sea water was acidified to the desired pH by the addition of concentrated hydrochloric acid. Air was then bubbled through the acidified sea water samples until constant pH readings were obtained. Stem segments were introduced into these sea water samples as in the previous experiment.

The results are summarized in Table II. As the hydrogen ion concentration of the sea water is increased, fewer stems regenerate during a given period. After 55 hours, 19 out of 20 stem segments regenerated at pH 8.08–8.04 (normal sea water), while only eight out of 20 stem segments regenerated at pH 7.05–7.04 (Table II, Exp. 3). The inhibitory effect was more pronounced as the pH was lowered, so that at approximately pH 6 no regeneration occurred. After 60 hours, four out of ten stems regenerated at pH 6.02–6.29, while at pH 5.93–6.08 there was complete inhibition (Table II, Exp. 1). The stems at the latter pH were kept in the experimental medium for a longer period (95 hours), at which time they still showed no signs of regeneration. As low as pH 6, the inhibitory effect is reversible, inhibited stems regenerating when returned to normal sea water. Below pH 6 the coenosarc tissue of many of the experimentals appeared to undergo gradual cytolysis and no recovery occurred. It may be noted that the oxygen concentration of the sea water samples was approximately the same for each experiment, and

that the concentration is in the range usually found in normal sea water. This rules out the possibility that inhibition was due to lack of oxygen.

DISCUSSION

Coenosarc fragments fail to regenerate when they are inserted into capillary glass tubing. A similar effect was demonstrated by Barth

TABLE II

The Effect of Increased Hydrogen Ion Concentration on the Regeneration of Tubularia Stem Segments

Medium	Initial pH	Final pH	Initial O ₂ cc./l.	Final O ₂ cc./l.	Number of regenerants
Experiment 1. Observed at 60 hours 10 stems at each hydrogen ion concentration					
Normal sea water	7.93	7.88	4.4	4.2	8
Acid S.W. + NaHCO ₃	7.89	7.94	4.0	4.1	7
Acid S.W. + NaOH-NaHCO ₃	7.87	7.42	4.4	4.1	7
Acid S.W. + NaOH	6.02	6.29	4.0	4.2	4
Acid S.W. + McIlvaine's	5.93	6.08	4.4	4.1	0
Acid S.W. + McIlvaine's	5.44	5.90	4.2	4.1	0
Experiment 2. Observed at 72 hours 10 stems at each hydrogen ion concentration					
Normal sea water	7.89	7.77	4.6	5.0	9
S.W. + HCl	6.77	7.23	4.6	4.9	7
S.W. + HCl	6.71	7.08	4.7	4.9	6
S.W. + HCl	6.57	7.19	4.6	4.8	6
S.W. + HCl	6.39	6.84	4.6	4.8	5
S.W. + HCl	4.88	5.70	4.8	4.5	0
S.W. + HCl	3.28	3.26	4.8	5.0	0
Experiment 3. Observed at 55 hours. 20 stems at each hydrogen ion concentration					
Normal sea water	8.08	8.04	4.6	4.6	19
S.W. + HCl	8.00	7.93	4.8	4.8	16
S.W. + HCl	7.91	7.74	4.7	4.7	14
S.W. + HCl	7.82	7.76	4.7	4.7	15
S.W. + HCl	7.62	7.56	4.7	4.7	11
S.W. + HCl	7.05	7.04	4.7	4.7	8

(1938a) using cut stems with perisarc, where covering of the cut end with glass tubing was sufficient to cause inhibition. The capillary walls decrease the diffusion rate and this might cause the oxygen tension of the sea water to fall below the level required for regeneration. In addition,

acid metabolites would accumulate in the sea water surrounding the tissues. Either one of these factors, or their combination, might cause the inhibition.

When the coenosarc fragments are placed in the tip of a capillary so that half of the tissue surface is covered by the capillary, while the other half is exposed to the sea water in the dish, regeneration always occurs at the exposed surface. Thus, the new polarity relationships developed by the homogeneous mass of tissue may be strictly determined as the result of a differential exposure to the environment. The differential exposure probably consists of two gradients, a gradient of oxygen tension, with the high end at the open end of the capillary, and a gradient of acid metabolite concentration, with the high end inside the capillary.

That oxygen plays a necessary role in regeneration has been clearly elucidated by other investigators. There is a threshold of oxygen tension below which regeneration will not occur, while above this threshold, the rate of regeneration is a function of the oxygen tension (Barth, 1938a). Miller (1937) obtained a reversal of polarity of *Tubularia* stem segments when the proximal cut end was bathed with oxygen and the distal end was bathed with nitrogen. Here, since the active bubbling of oxygen and nitrogen would remove waste products at the cut ends, the effect of oxygen was probably not vitiated by the inhibitory action of accumulated acid metabolites. The effectiveness of oxygen in regeneration is, however, limited by the inhibitory action of accumulated acid metabolites. This view is supported by the following evidences.

Most of the coenosarc fragments placed in the tips of capillaries regenerate in the direction of the exposed surface, despite the presence of a large oxygen bubble inside of the capillary. It may be assumed that the oxygen supply inside of the capillary was at least equal to that provided by normal sea water, so that regeneration should occur inside of the capillary. Since waste products presumably accumulate inside of the capillary, but are removed at the free end, it is indicated that the polarity of the regenerants was determined by the differential exposure to metabolic wastes. Thus, when there are no appreciable differences of oxygen tension at localized portions of the coenosarc fragments, a differential of the concentration of waste products may determine the polarity of the regenerants.

Coenosarc fragments inserted in glass capillaries do not regenerate when an oxygen bubble is placed on one side of the fragment and a nitrogen bubble on the other side. Failure to regenerate is probably due to the accumulation of acid metabolites, for again, sufficient oxygen was apparently available for regeneration to occur under normal conditions.

This interpretation is supported by the experiments of Rose and Rose (1941) in which it was demonstrated that regeneration is inhibited despite the presence of an oxygen bubble, when a cut end of a *Tubularia* stem is inserted into a tight-fitting cap. If the cap is loose-fitting, regeneration may occur.

Further evidence is offered by the experiments in which oxygen bubbles were introduced into stem segments. The coenosarc tissue was removed from the distal half of *Tubularia* stem segments. An oxygen bubble was then introduced into the empty half of the perisarc, and the cut ends were ligated. Ninety per cent of these stem segments failed to regenerate in running sea water, despite the presence of the large oxygen supply. This inhibition was probably caused by the accumulation of waste products. The stems which did regenerate, formed the hydranth at the distal end of the segment. Rose and Rose (1941) injected oxygen gas into the coelenteron of *Tubularia* stem segments and then ligated the ends. Twenty-one per cent of the stems regenerated when they were kept in running sea water, whereas none of the stems regenerated when they were kept in still water. Their results are understandable if waste products are removed more rapidly in an actively circulating medium.

The extent to which acid metabolites may accumulate as a result of capping is demonstrated by the change in pH of the sea water contained in glass tubing to which coenosarc fragments and stem segments had been added. In addition to inhibition of regeneration, there is a lowering of the pH of the sea water. With coenosarc fragments, the average pH of the sea water dropped from 7.96 to 7.11 after 96 hours, while with stem segments the average pH dropped from 7.96 to 6.90.

The acid metabolites probably cause inhibition by increasing the hydrogen ion concentration of the medium. The inhibitory action of increased hydrogen ion is demonstrated by the results of the experiments in which the pH of the sea water was regulated with acid, base, and buffer. The ability of *Tubularia* stem segments to regenerate is a function of the hydrogen ion concentration of the medium. At the oxygen tension of normal sea water, there is a range of pH in which regeneration is retarded, and a critical pH below which regeneration will not occur. The critical pH is probably related to the oxygen tension. Inhibition of stem regeneration in glass tubing occurred at a higher pH (pH 6.90) as compared with stems kept in open dishes (pH 5.93–6.08). This difference may be accounted for, if, in the glass tubing, a lowering of the oxygen tension of the sea water occurred concomitantly with the accumulation of acid metabolites. This would mean that the oxygen tension of the sea water required for regeneration is a function of the hydrogen

ion concentration. If this is true, it is suggested that the interrelationship of these factors in influencing *Tubularia* regeneration may be worked out in detail by studying regeneration in sea water at varying oxygen and hydrogen ion concentrations.

SUMMARY

Environmental factors effecting regeneration and the origin of polarity in regeneration were investigated. The experiments were performed using coenosarc fragments and stem segments of *Tubularia crocea*.

Regeneration was inhibited when coenosarc fragments and stem segments were inserted into glass tubing. Concomitant with the inhibition, there was a measurable drop in the pH of the sea water contained in the tubing.

Coenosarc fragments inserted into the tips of capillaries regenerated at the exposed surface of the fragments. The same polarization resulted in most cases, even when large oxygen bubbles were introduced into the capillaries.

Coenosarc fragments placed in capillaries, failed to regenerate, when an oxygen bubble was placed on one side of the fragments and a nitrogen bubble on the other side of the fragments.

When oxygen bubbles were introduced into the emptied distal portions of *Tubularia* stem segments, and the cut ends of the stems were ligated, only ten per cent of the stems regenerated.

All of these results support the view that the effectiveness of oxygen in regeneration may be limited by the inhibitory action of accumulated acid metabolites.

Acid metabolites probably cause inhibition by increasing the hydrogen ion concentration of the medium, for at the oxygen tension of normal sea water, there is a range of pH in which regeneration is retarded, and a critical pH below which regeneration will not occur.

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FURTHER STUDIES IN THE PHARMACOLOGY OF THE HEART OF *CANCER MAGISTER* DANA

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In earlier work (Davenport, 1941), it was found that there is a marked similarity between the effects of nicotine and acetylcholine on the isolated heart of *Cancer magister*. On the basis of this similarity, a hypothetical mediation mechanism was proposed for the decapod heart, and it was suggested that the effects of acetylcholine might be nicotine-like at the ganglion and muscarine-like at the neuro-muscular junction, since it had been demonstrated that the effects of acetylcholine are blocked by the administration of atropine (Welsh, 1939*a*, 1939*b*, Davenport, 1941). It became apparent that further tests with a number of sympathomimetic drugs and blocking agents would be of interest.

The technique of isolation and perfusion was similar to that previously used with one modification which should be mentioned, since it was found to give more uniform preparations. The heart was left attached by its ligaments to a polygonal piece of the carapace, which was clamped vertically in a fixed position; the posterior tip of the heart was tied off with a thread and attached to a light isotonic lever, and the perfusion fluid introduced via the aperture left by the removal of the descending vessel. In this way a minimum of manipulation was necessary after removal of the viscera.

The effects of the drugs that have been tested on the *Cancer* heart may be seen in the following table:

Drug	Response
acetylcholine	+
acetylcholine after eserine	++
atropine	○
acetylcholine after atropine	○
nicotine	+
nicotine after atropine	+
curare	○, -
acetylcholine after curare	○ or freq. - amp. +
muscarine	+
muscarine after atropine	○
doryl	+
doryl after atropine	+
mecholyl	+
mecholyl after atropine	○

+ = excitation, - = inhibition, ○ = no effect.

Previously it had been found, as observed in various arthropod hearts by other workers, that acetylcholine is excitatory, that treatment with eserine "sensitizes" the heart to acetylcholine presumably through its anticholinesterase activity, that atropine in low concentrations has little or no effect but that it abolishes response to acetylcholine. As has been stated, it had also been determined that nicotine has a marked excitatory effect on the heart resembling the effect of acetylcholine.

It next seemed of interest to determine whether atropine would have any effect on nicotine excitation. In a number of tests it was found that the response of the heart to nicotine 1:50,000, a characteristic increase in amplitude and frequency, was in no way modified by previous treatment with atropine 1:20,000 for twenty minutes, nor did atropine 1:20,000, when introduced immediately after the response to nicotine appeared, in any way alter the response.

EFFECTS OF CURARE

In several tests curare 1:5,000 had no observable effect on either amplitude or frequency. A heart with a slightly irregular beat was regularized by treatment with curare 1:400 and this regularization was accompanied by a decrease in frequency. Continued perfusion with curare brought about periodic stoppage in diastole (Fig. 1*A*). Carlson (1922) noted this cessation of rhythm in the crustacean heart after treatment with high concentrations of curare, but also observed a primary stimulation, which was not noted in the present tests. He states that after complete cessation of the rhythm, the heart remains excitable to direct stimulation.

Previous treatment with curare greatly affects the response to acetylcholine. A heart was perfused for approximately half an hour with curare 1:5,000, which had no visible effect. Treatment after this time with acetylcholine 1:10,000, a concentration which in the fresh heart produces a great increase in amplitude and frequency, can be seen (Fig. 1*B*) to have increased the amplitude only very slightly and the frequency not at all.

In a number of tests the characteristic effects of acetylcholine were not abolished but were profoundly modified by previous treatment with curare. A heart was treated for approximately twenty minutes with curare 1:5,000. Introduction of acetylcholine 1:100,000 brought about stoppage in diastole for a brief period. The curarization was then continued for a few minutes and acetylcholine 1:10,000 introduced. A brief period in diastolic stoppage was followed by an amplitude effect

characteristic of acetylcholine, but by a marked decrease in frequency (Fig. 1C).

This is in marked contrast to the behavior of the vertebrate heart in which abolition or modification of the response to perfused acetylcholine or to vagal inhibition by previous curarization has not been reported.

In a single test in which a heart had been treated for thirty minutes with curare 1:5,000 and in which the response to acetylcholine had been all but abolished, nicotine 1:10,000 produced a marked increase in frequency and amplitude. The response to this concentration of nicotine was not as striking, however, as it would have been in a fresh heart.

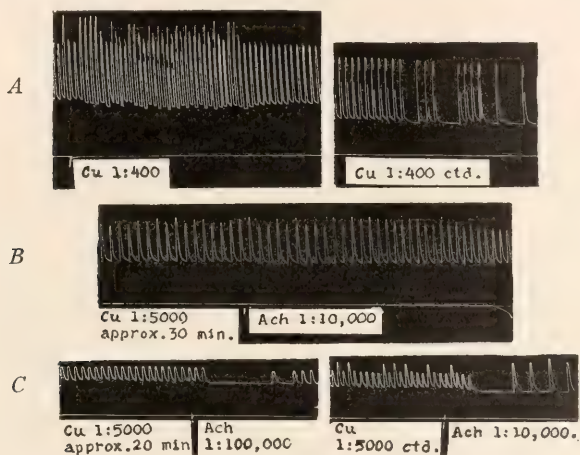


FIG. 1. A. Effect of curare 1:400. B. Effect of acetylcholine 1:10,000 after treatment with curare 1:5,000 for approximately 30 minutes. C. Effect of acetylcholine 1:100,000 and 1:10,000 after treatment with curare 1:5,000 for approximately 20 minutes.

Further tests should be run with this combination of drugs to determine whether curarization has an effect on the response of the heart to nicotine.

EFFECTS OF MUSCARINE

Concentrations of muscarine of 1:100,000 have a stimulatory effect on the heart of *Cancer* and increase frequency and amplitude without a rise in level of the recording. Concentrations as high as 1:5,000 (Fig. 2A) produce a marked increase in amplitude and frequency with an accompanying rise in level, but are apparently toxic, since slowing and irregularity of beat appear after perfusion for a short time. This is very similar to the effect of nicotine.

In a heart which had been treated with atropine 1:10,000 for fifteen minutes the response to muscarine 1:5,000 was completely abolished.

EFFECTS OF DORYL (CARBAMINOYLCHOLINE)

Bonnet (1937) observed the effects of the sympathomimetics, doryl and mecholyl, on the heart of the crayfish while investigating the antagonism between strychnine and acetylcholine in these animals. Mecholyl was found to accelerate the crayfish heart while doryl in the concentrations used retarded it. Bonnet's results are, however, not strictly comparable to the present work since his drugs were applied directly to the heart *in situ* with nervous connections intact.

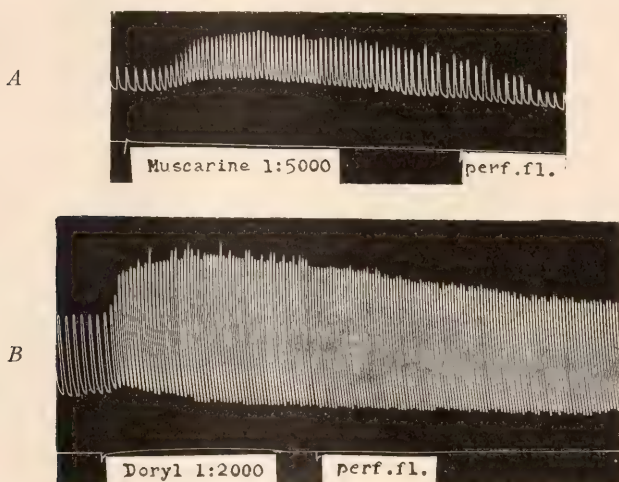


FIG. 2. A, Effect of muscarine 1:5,000. B, Effect of doryl 1:2,000.

In the heart of *Cancer* doryl 1:1,000,000 increases frequency and amplitude. This effect becomes more marked as higher concentrations are used. In no tests, however, even when concentrations as high as 1:2,000 were used (Fig. 2B) was it found possible to produce the marked increase in tone resulting in systolic tetany, characteristic of high concentrations of acetylcholine or nicotine.

In a number of tests it was found that previous treatment with atropine 1:10,000 had no effect on the response to high concentrations of doryl (1:2,000). In one test, however, a heart responded to doryl 1:1,000,000 with an increase in frequency and amplitude. Treatment with atropine 1:10,000 abolished this response when the doryl was

reintroduced, but failed to abolish the response of the heart to doryl 1:10,000.

EFFECTS OF MECHOLYL (ACETYL-BETA-METHYLCHOLINE)

This drug also has a strong excitatory effect on the *Cancer* heart, but in a test with as high a concentration as 1:1,000 no rise in the level of the recording was observed.

In a heart which had responded to mecholyl with a great increase in frequency and amplitude, treatment with atropine 1:10,000 for fifteen minutes completely abolished any response when the mecholyl was reintroduced.

DISCUSSION

Since the classical work of Carlson (1904) it has been thought by many that the heart-beat of arthropods in general may be initiated and controlled by the cardiac ganglion. This mechanism has been considered as a possibility in the heart of Crustacea by many workers. It has often been demonstrated that transection of the ganglionic trunk immediately stops the decapod heart; great care must be taken in the introduction of a cannula for this reason.

A hypothetical mediation mechanism was proposed (Davenport, 1941) by which acetylcholine might have a nicotine-like action on the controlling ganglion and a muscarine-like action at the neuro-muscular junction. After an examination of the above experimental data, Dr. Welsh has suggested that in this mediation mechanism the modes of action of acetylcholine at the two points be reversed.

The effects of acetylcholine, nicotine, and muscarine on the beat of the heart are seen to be extremely similar. It is known that nicotine has as a primary action the stimulation of ganglion cells, and in the crab heart it apparently excites the intrinsic pacemaker. If acetylcholine and muscarine also excite the ganglion but via a muscarinic receptor-mechanism, then abolition of the effects of the two drugs by atropine may occur at this point. It would seem that mediation at the neuro-muscular junction beyond the spontaneously active ganglion may be by the nicotine-like action of acetylcholine, because atropine has no effect on the response of the heart to nicotine and because atropine alone has no effect on heart-beat. In no tests with atropine alone in low enough concentrations to be of physiological significance has it been possible to demonstrate any marked effect on rate or amplitude of contraction.

Curare abolishes the nicotine-like effects of acetylcholine but not its muscarine-like ones. The tests in which curare abolished beat altogether

are additional evidence that mediation at the neuro-muscular junction is by the nicotine-like action of acetylcholine. However, the tests in which low concentrations of curare abolished the excitatory effects of acetylcholine do not exactly agree with the hypothesis that mediation at the ganglion is by the muscarinic action. Under these conditions curare must prevent acetylcholine from altering the spontaneous activity of the ganglion, in which case the receptor-mechanism at that point would have to be considered both nicotine-like and muscarine-like. To fit in with the hypothesis curare should block the stimulatory effects of nicotine on heart-beat; unfortunately a single inconclusive test was run with these two drugs.

Doryl and mecholyl were tested to determine whether the heart would respond differently to a drug having both nicotine-like and muscarine-like actions than to one having muscarine-like alone. No marked difference in response could be demonstrated; however, it could be seen that the effects of doryl are less strongly antagonized by atropine than those of mecholyl, which are as in the vertebrates completely abolished.

It is apparent that studies must be made of the effects of drugs on the spontaneous activity of the intrinsic ganglion and the consequent behaviour of the heart.

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SOME NEUROHUMORAL EVIDENCE FOR DOUBLE INNERVATION OF XANTHOPHORES IN KILLIFISH (*FUNDULUS*)

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Many experimental investigations during the last dozen years indicate (see Parker's review, 1940a) that the dermal melanophores of several teleost fishes, including *Fundulus*, are subject to the influence of both pigment-dispersing and pigment-concentrating autonomic nerve fibers and that such influence is exerted through chemical mediators. The later contributions of Chin and Li (1941), Healey (1940), Parker (1940b), Parker (1941), and Parker and Rosenblueth (1941) in general strengthen the case both for double innervation of the effectors studied and for chemical transmission from the nerve endings to the melanophores.

Work on other chromatophores has remained relatively scanty. In the same kind of fish one would expect to find, as Parker (1932, 1940) has assumed for the xanthophores, the same type of nervous and humoral regulation as in the melanophores. Results reported by Smith and Smith (1935) and Parker (1937) on the squirrelfish and by Dalton and Goodrich (1937) on the paradise fish support this view as regards erythrophores. Double innervation of xanthophores is implicit in Abramowitz's (1936) consideration of the neurohumors that apparently govern them. I had found that both nerves, for pigment concentration, at least, and humoral substances control xanthophore responses in the killifish *Fundulus heteroclitus* (Fries, 1931). Giersberg (1932), using partly the same methods, could demonstrate pituitary but no nervous control of the xanthophores and erythrophores in the minnow *Phoxinus*, a form in which melanophores do receive nerves, probably both dispersing and concentrating. Vilter (1939) has held that the xanthophores in another cyprinid (*Carassius*) lack nervous regulation. The possibility that certain chromatophores in some species have *single* (pigment-concentrating) innervation is suggested by work on elasmobranchs (Parker, 1936).

The present paper¹ reports further results pertaining to the nature

¹ This paper owes its origin to a small project that had attracted the interest of Dr. G. H. Parker, but which he kindly ceded to me. The work was done during two early-September stays at the Marine Biological Laboratory, Woods Hole, Mass. To the helpful officers and personnel of that institution I acknowledge grateful indebtedness.

of the nervous mechanism earlier found to control the xanthophores in *Fundulus*.

DESCRIPTION OF EXPERIMENTS

The experiments consisted basically of caudal cuts similar to those Parker (1934) devised in studying catfish melanophores. The initial, or "primary," cut (1 in Fig. 1) severed two or three fin rays with their contained nerves, etc., and with their associated interradiar tissue; this cut was made at or near the distal edge of the scale-covered zone or else just proximad of the main vascular arc connecting the axial caudal blood vessels with the mostly radially arranged peripheral ones of the fin. Distal to any such cut, a yellower as well as a darker band quickly appears, characterized by pigment dispersal in both yellow and black chromatophores (Fries, 1931).² After a week or more, when this primary band had faded by gradual pigment concentration in consequence of the fish's response to a white or pale-blue bottom, a pair of "secondary" cuts (2 and 2' in Fig. 1) through an appropriate span of fin-ray branches established new yellow and dark denervated bands flanking the distal portion of the primary band. These primary and secondary cuts were carried out on males and females, about 6-8 cm. long, mainly of the striped killifish, *Fundulus majalis* Wahlb., which has more distinct xanthophores than the common killifish, *F. heteroclitus* L. Externally painted glass bowls (mostly 10-inch culture dishes) with running sea water served to contain the fish and to call forth their chromatic responses to colored backgrounds.³

In Parker's (1934) and Matsushita's (1938) experiments on catfish melanophores, pigment dispersal in secondary bands spread into previously faded primary bands but not into intact control bands; also, responses of denervated bands to black or white bottoms occurred by gradual spread from adjacent innervated areas. Blood-borne hormones being found innocent of such spread effects, it is held (1) that pigment-dispersing nerve fibers aroused to prolonged activity by the cut are responsible for the dispersal, and (2) that these nerves act by secreting a lipid-soluble dispersing "neurohumor" that can diffuse slowly even to rather distant melanophores.

Do the xanthophores of *Fundulus* behave similarly, calling for like interpretation? Among a group of 26 *F. majalis* given secondary cuts

² For an account of the disposition of the yellow pigment in the xanthophores of *Fundulus*, see Warren (1932).

³ The paints used were Carmote Colorquic white (for some dishes turned just perceptibly bluish with a trace of prussian blue), the M. B. L.'s acid-resistant black, B. Moore & Co.'s Utilac medium blue and Utilac yellow.

after the fading of successful primaries, 15 showed indications, at least once, of some spread of the yellow effect produced in bands II and II' into the I_d half of the primary band, yet not into the N areas (Fig. 1). Several of these afforded beautifully clear demonstrations. Among them were two given a third secondary cut (2_c in Fig. 1B and C) to provide an equal innervated band (N_{cd}) as simultaneous control; no trace of

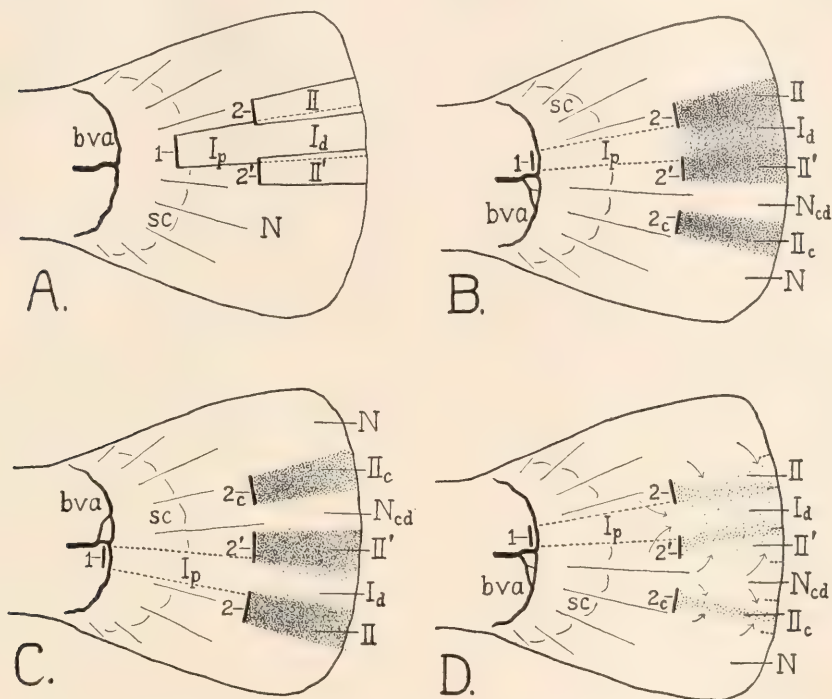


FIG. 1. Diagrams of *Fundulus* tail fin. A. Showing denervated bands produced by transverse incisions (all cutting radial blood vessels). B. Showing similar bands, but including one more for control, and showing primary incision that avoids blood vessels; with stippling to indicate a good example of pronounced affirmative results. C. Ditto; but stippled to indicate minimum affirmative results. D. Ditto; but stippled to indicate loss of pronounced effect (B) through general pigment concentration, arrows marking progress of fading of yellow zones, or encroachment of pigment concentration from innervated zones. 1, primary cut, and I , corresponding primary band, with proximal (I_p) and distal (I_d) portions distinguished; 2 and 2', secondary cuts, and II and II', corresponding secondary bands; N, normal innervated regions; 2_c , additional secondary (control) cut, and II_c , corresponding secondary band, providing N_{cd} , distal normal control band; bva, blood-vessel arc of main arteries and veins; sc, distal limit of scales. Cut 1 was made dorsal to the main axial caudal vessels according as collateral supply to the arc afforded best clearance; the position of II' and the control bands was made to correspond inversely.

spread of the yellow effect from the adjoining bands (II' and II_c) appeared in the control bands. In these two best cases and a few others the secondary cuts were made two weeks after the primary, by which time there was extra assurance of complete degeneration of the primary-band nerves. But, because as many as 11 of the 26 showed only negative or dubious, faintly affirmative results, I took the next opportunity to carry through experiments on another set of 25 *F. majalis*, each with the self-contained control afforded in band N_{cd} by cut 2_c (Fig. 1B and C).⁴

Sleek, sound fish were selected after at least two days of acclimatization to the laboratory aquaria, i.e., after the wave of deaths that in this species often follows seining and transport. Unlike the earlier lot, the fish were fed almost daily with minced clams. The unregulated temperature of the running sea water was close to 20° C.; occasionally the fish were exposed to warmer standing water. For all tail-cutting operations the fish were subjected to cold anesthesia in an ice-and-water mixture. The best tools for the cuts had proved to be chisel-like slivers, $\frac{3}{4}$ –2 mm. wide, of a Gillette-type razor blade, fastened to handles several centimeters long. The cuts were made against an enlarged glass stage under the low power of a dissecting binocular microscope after removal of any scales covering the surface to be penetrated. To assure from the outset minimum vascular differences between the innervated regions and the primary denervated band, all primary cuts, usually through two or three fin-ray bases, were made just proximad of the blood-vessel arc and close dorsad or ventrad of the main axial vessels (Figs. 1B and C). Within the hour specific note was made in which rays and interrays the nerve supply was severed, as shown by dispersal in the chromatophores which was obvious in 10–30 minutes. Usually a recheck was made on the day after the cut. At this latter time the dark and yellow band was commonly less wide than originally. Some of this loss in width may be due to the eventual dominance of uncut concentrating fibers over the influence of less strictly radially distributed cut dispersing fibers (Mills, 1932). This narrower band after fading could be revealed as a pale band by putting the fish for a few minutes over a black bottom. It may then be considered fully denervated at least in regard to any dispersing fibers. The secondary cuts 2, 2', and 2_c (Fig. 1) were made equally long, with the same chisel, carefully oriented to adjoin the previously recorded primary band and to make bands I_d and N_{cd} equally wide; they were made eight days after the primary cut, except for five sets made six days after and six sets made ten days after. All critical determinations

⁴ Though I did not know of Matsushita's (1938) work when I first applied this method to three fish in my earlier lot of experiments, he had already devised and made use of the same expedient.

of the results in the primary and control bands were made with the binocular, the fish usually being held with bare fingers. The binocular was used, too, for most other observations of the state of the caudal xanthophores, and in every instance of possible doubt. Besides some daylight (two big windows, but dark interior), illumination was by two 75-watt daylight bulbs hung in white shades two and one-half feet above the bowls and four feet apart. Under these conditions the fish changed shade greatly in two minutes when shifted between black and white bottoms and showed definite macroscopic differentiation of dorsal yellow vs. not-yellow hues in 15–30 minutes on interchange of yellow and blue bowls.

The results for the second season resembled those of the first. Among the 25 fish, carried through to one or more observations following the secondary cuts, 14 yielded affirmative indications at least once of some spread of the pigment-dispersal effect from bands II and II' to the xanthophores in I_a but not from II' and II_c into N_{cd} (Figs. 1B and C); four more might be included in the same category except that the proximal part (I_p) of the primary band turned about as yellow as the distal part (I_d) when the secondaries (II and II') were formed; in three others the indications were doubtful, and the remaining four gave only negative results in the time available. One of the 25 did not withstand the operations and handling well enough to be examined for the results more than once, and the condition of the tails of several others at the inspection time was below standard. None of these poor specimens was included among the definitely affirmative 14 cases. The good cases included at least two that demonstrated the spread effect, confined to I_a , as diagrammatically as Fig. 1B. In the others of the 14, the effect was less pronounced, in some consisting solely of a slight transition of the xanthophore state within the edges of I_d toward the degree of dispersal characterizing the xanthophores of the adjacent II and II' bands (Fig. 1C).

Among the 14 fish were 9 that failed to give the same affirmative results at some other time or times of inspection. In this connection it is well to note that the yellow spread from bands II and II' into band I_a is rarely visible in killifish kept over a bottom inducing full pigment concentration in the xanthophores; and when it does appear, it disappears on further manipulation of the fish. Accordingly, the full count of affirmative results in both sets of experiments was obtained only by coaxing, as it were. Such "coaxing" meant that when the primary bands had faded in the fish kept in white or blue bowls, yellow bowls were substituted to induce reduction of the factors for pigment concentration throughout the tail fin. Thereby dispersal was promoted, especially in the denervated zones, and above all in the freshly formed secondary bands.

As a rule, though contrary to the indications of the obvious yellowing of the body, the innervated xanthophores in the tail fin generally got no further than the intermediate state while newly denervated xanthophores regularly attained maximum or near-maximum pigment dispersal (maintained for days over a yellow bottom).⁵ In case the innervated xanthophores did show too much dispersal, the fish were again placed in a white or blue bowl to induce some reconcentration in the innervated xanthophores for the sake of better contrast with the xanthophores in the secondary bands. Sometimes several shifts of the fish from color to color were necessary to obtain the balance between dispersing and concentrating factors in the primary band permitting demonstration of the spread into it of pigment dispersal excited in the flanking secondary bands. For their strong showing of the effect even my best examples depended upon favorable adjustment of bottom colors and of factors involved in handling or exciting the fish.

DISCUSSION OF RESULTS

Considering the whole trend of results as tallied for the two lots of fish, the evidence seems favorable to the hypothesis that xanthophore innervation includes dispersing fibers that secrete a dispersing neurohumor capable of spreading from cell to cell. In two other corroborative experiments a single flanking secondary band resulted in a spread of its yellowing effect into the faded primary band but not into the equally contiguous innervated region.

The failure of the xanthophores in intact regions to be influenced by adjacent denervated bands and their ability, not shared by denervated xanthophores, to show *prompt* adaptive response to white or blue ground color confirms the conclusion (Fries, 1931) that the xanthophores are supplied with concentrating nerves. In addition, the results in general, and particularly in their dependence on the "coaxing" procedure, accord with the view that these concentrating fibers secrete a corresponding neurohumor which, like the dispersing neurohumor, can reach more distant xanthophores by diffusing through tissue lipids (cf. Parker, 1940*b*, on melanophores). Excess of such a concentrating neurohumor, richly

⁵ That the yellow bowls generally failed even in the course of more than a day to evoke full or almost-full dispersal in the xanthophores of the tail fin was contrary to prior findings (Abramowitz, 1936, for this species), yet certainly typical of the fish in these experiments—irrespective of the year or of the food supply. All observations, with rare exceptions, were begun within a few seconds after the fish had been swimming undisturbed in the bowl. Thus there was too little time for excitement or other stimuli to induce much change in the xanthophores from the condition they had assumed. The incomplete response in the tail fin to the yellow bottom color remains unexplained.

produced in innervated parts of the tail during response to a white or blue bottom and spreading into the bands, may (especially if assisted by any substance carried in the blood) fully counteract the dispersing neurohumor everywhere except at the site of its active production and consequent greatest abundance in the secondary band. Thus applied, the hypothesis accounts for rather frequent negative results in the critical tests of cell-to-cell spread of a dispersing neurohumor.

Apparently less reconcilable with the hypothesis than simply negative results was the fact that increased yellowness (pigment dispersal), resembling the spread into the denervated band from bands II and II', appeared in the control band (N_{cd}) once in each four of the group of 25 fish. Among the four were three of the 14 that on at least one other occasion showed the spread only in the denervated band (I_d). We are concerned, therefore, not with four fish versus 14, or four out of a total of 25, but with four single instances of a phenomenon that failed to appear in more than 60 opportunities. Local equilibria of several pigment-motor factors might get mutually out of step more readily under the shifting conditions of changing bottom colors and incidental handling than with steady visual stimulation from a white bottom. This possibility may offer an adequate explanation of the four instances of yellowness of the innervated control band.

There are further reasons for holding to the interpretations involving antagonistic nerves and neurohumors. In many instances the fading of a primary yellow band obviously proceeded axipetally, i.e., beginning at the longitudinal edges, gradually extending inward, and becoming complete last in the axis of the band. In several other instances, when the intact zones became yellow after a yellow band had faded, redispersal in the band, lagging behind the dispersal occurring elsewhere, clearly took place by invasion from the edges to the axis. I could find no evidence for supposing that differences in the flow of blood, carrying typical circulatory hormones, were responsible for this mode of xanthophore change in the denervated band. When the blood flow throughout the band (cut proximad of the blood-vessel arc) was equal, as far as discernible, to that in adjacent innervated regions, axipetal change occurred as much as when the flow was obviously obstructed in bands made by distal cuts. Therefore, Vilter's (1939) explanation of lagging, axipetal band changes in terms of defective band circulation, assumed to limit renewal of local stores of intermedin, would be untenable applied to the present results. Instead, the axipetal changes in the bands conform perfectly with the view that the phases of xanthophore response depend upon different chemical and physical equilibria of the medium, that antagonistic pigment-motor nerve secretions enter into such equilibria, and that slow cellular

transmission of such secretions from active fibers is the limiting factor of xanthophore response in regions where central control of the nerves is interrupted and where there is at the same time insufficient blood-borne pigmentary hormone to swamp the effect of the nerve secretions.

Both lots of killifish supplied many examples of progressive fading of yellow secondary bands inward from the innervated edge, while progressive fading occurred along the edge next the primary denervated band only considerably later, after any prior yellow spread from the secondary bands into the primary band had been dissipated, and through gradual roundabout invasion from the innervated zones (Fig. 1D). The dual neurohumoral explanation is again wholly adequate. In particular the fading of either secondary or primary bands inward from adjoining innervated sectors argues for nervous production and cell-to-cell diffusion of a concentrating neurohumor.

Pigment concentration in all areas where the xanthophores exhibited any dispersal took place repeatedly as I held fish on the microscope stage for one-half to three minutes. On such occasions yellow bands, especially older ones, faded considerably, if not completely, while I watched. Sometimes, when the first inspection was limited to a few seconds, another look ten minutes later revealed that the same sort of fading had occurred and had not yet been reversed during the interval back in the yellow bowl. Frequently correlated with such quick fading of yellow bands and with equally quick concentration in innervated xanthophores was simultaneous stoppage or near-stoppage of blood flow into all sectors of the caudal fin. Resumption of circulatory inflow in the next minutes with more or less distention of veins throughout the tail was correlated with redispersal in the xanthophores that had just shown concentration, the redispersal appearing more markedly in newer bands and in innervated zones. The fact that this redispersal appeared least in old denervated bands accords with the hypothesis of a dispersing nerve secretion and the idea that activity in dispersing fibers excited by cutting diminishes after a time as the nerves degenerate (cf. Parker, 1941). Once dominated by the concentrating agents and caused to fade, such older denervated bands could not, according to the hypothesis, so quickly come to contain as much dispersing neurohumor as could innervated zones or newly excited bands.

The quick pigment concentration in both denervated bands and in innervated regions seems, on the contrary, to depend on what the blood brings. It failed to occur equally just next to rather new cuts in the fin where the blood with less effective collateral circulation was more obstructed than distally in new bands. When the circulatory change happened to be noticeably unequal in different sectors of the fin, then the

first, fastest, or greatest concentration was several times observed to occur in the xanthophores of the sector supplied at the moment with the strongest blood flow. The facts noted make it improbable that the quick pigment concentrations are due to lack of oxygen in blood (cf. Fries, 1931; Parker, 1938), which has not been found to have such a quick effect. Suspicion falls more naturally on adrenaline: it is well known to have not only vasoconstrictor action but pigment-concentrating effects on some chromatophores, and Abramowitz (1936) found that injections of an adrenal preparation evoked the concentrated condition in both innervated and denervated xanthophores of *Fundulus majalis*.

A few experiments involving primary and secondary bands, duplicating those for *F. majalis*, gave comparable results for the xanthophores of *F. heteroclitus*. Out of seven fish given secondary bands 10–15 days after the primary cut, one showed a good, and two showed some, spread of yellowing from the secondary bands into the primary between them. In two more the primary band turned yellow even in its proximal part; in the other two the results were doubtful. As far as may be judged on this basis, the xanthophores of both species of *Fundulus* evidently have identical innervation.

Though the chief recent work on teleostean melanophores, supporting the hypothesis of double innervation and of neurohumoral transmission, deals with the catfish, various evidence indicates that the melanophores of *Fundulus heteroclitus* are under essentially the same kind of nervous control (Parker, 1940a). It ought, therefore, to be possible to demonstrate in *Fundulus*, though Parker found the catfish better for the purpose, that a new dark band induces an adjacent denervated pale region to darken, a change consonant with the idea of an invading dispersing neurohumor. In my experiments on *F. majalis*, while primarily concerned with the xanthophores, I observed the melanophores enough to gain a well-grounded impression that, in this species, they demonstrate this about as often as the xanthophores. Unquestionably the melanophores in the faded primary band, not in the pale innervated control band, in several cases showed marked pigment dispersal between strongly darkened, flanking, secondary bands, especially next to their margins. No indications of the same phenomenon could be detected in some other cases. If double innervation and neurohumoral transmission be accepted for the killifish's melanophores on wider grounds, then the resemblance between its melanophores and xanthophores is another point in favor of accepting both concepts for the xanthophores.

What of possible alternatives? The pituitary must be considered. That it does indeed supply a hormone important for dispersing the pigment in *Fundulus* xanthophores, as suggested by analogy with *Phoxinus*

and by the results of injecting pituitrin (Abramowitz, 1936), will be shown in another paper being prepared for publication. No dispersing hormone, however, can of itself account for the spread of dispersal from the secondary bands exclusively into the distal part of the test band when dispersal is elicited in the secondaries, nor for the failures of the spread to appear at all. On the other hand, if this hormonal mechanism, probably plus an antagonistic one (adrenaline?), is thought of as co-existing with the humorally mediated double innervation, then the previously noted exceptional occurrence of dispersal in I_p as well as I_d (Fig. 1) puts little strain on the neurohumoral interpretation; such exceptions might well occur when a surplus of pituitary hormone coincides with so reduced a supply of the concentrating nerve secretion that the latter, though remaining dominant in intact regions, fails to dominate in any part of the denervated bands. Also, if comparatively reactive chromatophores in such fishes as *Fundulus* are delicately poised in relation to three or more different controlling substances, considerable variability of response is to be expected, so that other deviations from the standard spread effect would follow from excessive variations in the blood supply of the hormones or in the tone of the pigment-motor nerves.

The most plausible alternative to the thesis advocating dispersing nerves would assume co-existence of the pituitary dispersing mechanism, and perhaps a concentrating hormonal mechanism, with single innervation supplying or withholding pigment-concentrating impulses. It implies appropriate flexibility of behavior in denervated and intact chromatophores. But even if amplified to include chemical transmission from the concentrating fibers (Veil, 1936), it accounts less well than double innervation for those changes in primary and secondary bands that occur by invasion from the edges. It fails still more to account for the re-eliciting of a yellow band by recutting an already denervated but faded band (Parker, unpublished; cf. Parker, 1940*b* and 1941).

Altogether the experimental results and incidental observations, added to prior data, in my opinion support the conclusion that the xanthophores of *Fundulus majalis* and probably also *F. heteroclitus* are governed by a neurohumorally mediated reflex mechanism, including dispersing and concentrating autonomic innervation, as well as a longer-distance humoral mechanism with a pituitary dispersing hormone (presumed to be intermedin) and probably some concentrating hormone.

SUMMARY

In a majority of over 50 *Fundulus majalis* tested, the part of a previously faded primary denervated caudal band flanked by two new secondary bands showed pigment dispersal in its xanthophores, as if affected

by the stronger dispersal in those of the adjacent secondary bands. The effect appeared whether or not the radial blood vessels were cut by the incision that produced the primary band. Intact control bands, flanked identically, generally gave no sign of a like effect.

A few experiments on *F. heteroclitus* revealed comparable responses by its xanthophores.

These observations derived from the xanthophores were paralleled by some from the melanophores in *F. majalis*.

Characteristically, slow changes in the xanthophores in response to bottom color proceeded axipetally in primary bands, and they took place in secondary bands by an analogous invasion from the edge adjoining an innervated sector rather than from the edge of the primary band.

Quick pigment concentration apparently excited by handling, holding on the microscope stage, etc., appears in denervated bands as soon as in intact sectors.

These results, certain exceptions, and other available evidence together prompt the conclusion that the xanthophores of *Fundulus* are probably controlled by nervous and humoral mechanisms involving the following components in addition to a dispersing pituitary secretion and some concentrating hormone (adrenaline?): A. Double autonomic innervation, which includes (1) dispersing fibers, (2) concentrating fibers. B. Chemical mediation, the nerves secreting, respectively, (1) a dispersing neurohumor, (2) a concentrating neurohumor. C. Cellular transmission of these neurohumors to neighboring xanthophores.

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NOTES ON COLOR CHANGE AND PIGMENTARY INNER- VATION IN A GOBY, A WRASSE, AND THE PLAICE

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Integumentary color change differs among teleosts. In many species it is more strictly a change of shade, while in some the hue is also actively altered. The effectors of all such changes, the chromatophores, appear to differ, according to their kind and the species in which they occur, in their mechanism of control. Recent work on teleost melanophores, going beyond the accepted conclusion that they are innervated, stresses the role of hormones (primitive, accessory, predominant, as the case may be), of double innervation in at least some species, and of chemical (neuro-humoral) mediation of the nervous regulation. As for the erythrophores and xanthophores that may participate in color change, innervation seems established for some teleosts, not for others. A better unified understanding of these matters, amid so much apparent diversity, will depend on additional observation and experimentation.

To this end, exploratory study of several species of teleosts common near Fiskebäckskil¹ yielded results which, though in part incomplete or tentative, are recorded in this paper.

METHODS

All the fishes were kept in running sea water, which stayed within two degrees of 17° C., and were fed every few days on minced mussels. Glass bowls, about 25 cm. in diameter, coated externally with black, white, bright rich yellow, pale blue, or vivid red paint were used to elicit the color responses. Ample daylight was admitted through large windows. All macroscopic determinations of color or adaptation were comparative, the fish being viewed simultaneously over like backgrounds, mostly white bowls or pale grayish sand. For microscopic examination of the chro-

¹ At the Kristineberg Zoological Station (of the Royal Swedish Academy of Science), on the west coast of Sweden. Accorded every facility and ready aid in supplying my wants, I owe Professor Einar Lönnberg, the director of the Station, and Dr. Gunnar Gustafson, the manager, particular acknowledgment of their hospitality.

matophores, chiefly in the tail fin, I used a dissecting binocular. Cracked ice in water served as anesthetic for the operations.

GوبيUS MINUTUS Pallas

This goby ("common goby") has plentiful red and small yellow chromatophores, besides melanophores and guanin-containing elements,² which on the trunk are commonly joined into chromatic complexes, as detailed by Ballowitz (1913*a*, 1913*b*). In the caudal fin occur prevalently only separate red, yellow, and black pigment cells.

Meyer (1931) has described how this fish attains, rather slowly, a color well approximating that of several experimental backgrounds. My tests of its responses to black, white, red, and yellow essentially confirmed her results as regards the rate, nature, and extent of the adaptations to these colors, except that I found, in the tail fin, poorer differentiation of xanthophore response; their pigment was not concentrated much in response to red, as compared with the dispersal evoked by yellow, and usually did not, even in nine days, become completely concentrated in response to white. Meyer did not mention blue. I found that blue evokes a response distinct from those to red and yellow (besides black and white). For example, two lots of five and four fish in two days over blue and yellow, respectively, showed macroscopic differentiation, not only slightly of shade but clearly of hue. This difference was half lost in forty-five minutes over a neutral ground of sand and reversed, as verified microscopically in the tail, in six hours over the reversed colors, yellow and blue. The blue response involves pigment concentration in both erythrophores and xanthophores, and a more nearly intermediate condition in the melanophores. Were these the only factors, there would be no difference from Meyer's results with gray; but as the skin is chromatically more complex (e.g., iridocytes and iridosomal combinations are abundant on the trunk), the goby can turn truly bluish.

Morphological color change became obvious sooner than anticipated, especially in young fish less than 4 cm. long. After five days over white, considerable degeneration of melanophores and extrusion of melanin through the epidermis (cf. Odiorne, 1936) was seen to have occurred.

In response to changed bottom color, the melanophores usually changed twice as fast as the erythrophores, yet the latter could show concentration through more than half their full range in half an hour. The more vaguely outlined xanthophores seemed slower. Meyer (1931) was impressed by the slowness of complete concentration or dispersal of the pigment, even in the melanophores, and by the relatively quick start of

² Compare Odiorne's (1933) description of guanophores and discussion of the terms guanophore and iridocyte.

the change, even in the erythrophores. She supplied convincing evidence of the humoral control suggested by the slowness, but did not do likewise for the direct nervous control expected on the basis of earlier work on other teleosts (e.g., von Frisch, 1911 and 1912) and indicated by the initial rapidity of the change. Unqualified reliability of the latter indication seems open to question, however, for humorally activated pigment concentration in a denervated band may occur very quickly (Fries, 1942).

More direct evidence of nervous control is supplied by denervating operations in which two or three rays of the caudal fin with their inter-radial tissue were severed. In gobies kept in white bowls or over pale grayish sand, such cuts, whether at the distal border of the caudal scales, or more proximally, or out in the fin, regularly provoked slow pigment dispersal in the melanophores and erythrophores, and less obviously in the xanthophores, throughout a band distal to the cut and enclosing the cut rays and their branches. The dispersal appeared first in the melanophores, evidently reached the maximum for the erythrophores in about six hours, and took a little longer in the xanthophores. Subsequently the bands tended to fade, but in the case of 3-ray bands did so only slightly, although full concentration prevailed in the intact sectors in response to a light bottom (e.g., pale sand for ten days, then white for four). In the 2-ray bands, five days over sand plus five over white sufficed for the pigment of the melanophores and erythrophores to attain typically a concentration almost equal to that evoked in the intact parts of the fin. But in fish transferred to red for five days following five over sand, the red, yellow, and black pigments were dispersed to the same extent in such 2-ray bands as elsewhere in the fin. Moreover, when white-adapted fish with almost wholly faded bands were transferred to red for several days, the band pigments became redispersed to match the chromatophores in the intact regions. The redispersion in the band erythrophores and melanophores appeared in some of the fish to lag behind the dispersal outside the band. In case of the reverse transfer from red to white of fish with the band chromatophores in the same state as in other sectors, the resulting general concentration of pigment did proceed more slowly in the band. This lagging concentration in the denervated band was clear as regards both erythrophores and melanophores, but less sure as regards the xanthophores. The lag was verified in each of three fish tested as late as nineteen days after the denervating cut. The inability of the chromatophores in the caudal bands, despite fully restored circulation, to show normal concentration in response to visual stimulation is evidence that there is direct pigment-motor innervation involving concentrating nerves. The persisting capacity for some visually-induced response is evidence of humoral control.

Signs of beginning nerve regeneration in the denervated band were noted in a few gobies examined twenty days after the nerve section. By the twenty-eighth day no lag in the responses of the band chromatophores behind the innervated chromatophores could be detected, and virtually complete regeneration was further indicated in each of three gobies in which I recut the fin through the old scar with the result that a strongly renewed full-length band was displayed in six hours by all three types of chromatophores (cf. Parker and Porter, 1933, and Abramowitz, 1935).

Thus far there is close resemblance to the chromatophores of the killifish and the catfish. It suggests that the goby's melanophores and erythrophores, if not also the xanthophores, may have the same sort of neurohumorally mediated double innervation that is strongly indicated for the melanophores of *Ameiurus*. To throw some light on this possibility, I induced the formation of three secondary caudal bands, two flanking an old primary band and the third setting off an equivalent innervated band for control, as described in an earlier report on *Fundulus* (Fries, 1942). The secondary cuts were made two weeks after the primary, which presumably allowed quite ample time for complete nerve degeneration in the primary band. More or less convincing results appeared in the 11 fish in which the three secondary bands were successfully established. In the distal portion of the primary band (flanked by the secondary bands with more extreme pigment dispersal) the melanophores and erythrophores in all 11, and the xanthophores in two or more, showed a dispersal of their pigment that was not matched in the proximal portion of the primary band and not shown at all in the control band. So stated, the effect prompts explanation in terms of Parker's hypotheses—i.e., as due to a slow diffusive spread into adjacent regions of neurohumoral material secreted by recently cut and still excited dispersing nerves in the secondary bands and to the coincident dearth of concentrating factors in the denervated primary band as compared with the innervated control band. But the pigment concentration in the primary band was not fully equal to that in the innervated regions when the secondary cuts were made; and there was already a slight tendency in several cases to less concentration throughout the distal part of the fin. Still, it seemed safe to conclude that these two possibly misleading conditions were not responsible for all the effect observed.

Accepting the results, accordingly, as having some neurohumoral significance, one may ask why they should be taken as evidence for *dispersing* neurohumors and nerves: why not regard them rather as evidence of the assumption of a resting state (dispersal) by the chromatophores when their concentrating nerves are cut and when the supply of concentrating neurohumor diffusing into old bands from regions with uncut

nerves becomes exhausted in the part of a primary band isolated by the secondary bands? Rejection of this alternative stems first from other fish where fuller work supports belief in dispersing fibers (Parker and Rosenblueth, 1941) and in a different concept of chromatophore rest (Parker, 1940*b*). I would add an observation several times verified on *Gobius*. Handling pale fish provoked rapid darkening and reddening; i.e., the innervated caudal melanophores and erythrophores showed considerable dispersal of pigment in half a minute, while band chromatophores failed to show corresponding dispersal. This phenomenon can be explained better in terms of nerves than of hormones brought from a distant gland and supplied to a week-old band as richly as to the rest of the fin. It is better ascribed to the activity of dispersing nerves (cf. Parker, 1940*c*) than to inhibition of concentrating nerves.

When making this study of *Gobius minutus*, I was unaware of Vilter's related work (1938, 1939*a*, 1939*b*). Vilter obtained certain results with *G. lota*³ and other teleosts that he took to be in conflict with Parker's reactivation of blanched bands, viz., he was unable to get renewal of pigment dispersal in faded denervated zones by recutting (Vilter, 1938). Besides the few already mentioned tests of nerve regeneration at three and four weeks, I recut the nearly faded primary band about midway of its length in two gobies. In both of them the primary band melanophores and erythrophores distal but not proximal to the new cut showed renewed pigment dispersal. Since the recutting was done fourteen days after the original cuts (Parker, 1941, Osborn, 1939), when degeneration of the original nerve endings was supposedly complete and regeneration could hardly have begun, the results are an incentive to further tests of the non-reactivation stressed by Vilter. His negative results seem of doubtful significance until confirmed in relation to other variables (Parker, 1941), such as the extent of interference with circulation. Vilter (1938) further observed that pigment dispersal was "permanent" in the middle one of three cut rays of the dorsal fin. My observation that 3-ray caudal bands fade less than 2-ray bands is comparable. Considered with due regard for the importance of intermedin as an additional factor for pigment dispersal, both observations are in harmony with those made on bands of various widths in *Fundulus* (Parker, 1940*a*, p. 183) and hardly contradict the hypothesis of competing nerve-secreted humors. Altogether, Vilter's results as published agree with the work of Parker and others on *Fundulus* in virtually all of many particulars, with the one noteworthy exception of the non-reactivation already discussed. Vilter's explanation is clouded by his assumption that xanthophore

³ This species seems to be the same as *G. ophiocephalus* Pallas.

change is necessarily indicative of intermedin (Vilter, 1939b) and not subject also to direct nervous control such as is involved in *Fundulus* (Fries, 1942) and probably in *Gobius minutus*. Thus it appears premature to reject for *Gobius* the hypothesis that its melanophores, erythrophores, and possibly xanthophores are governed by dispersing innervation in addition to concentrating nerves and pituitary secretion.

LABRUS OSSIFAGUS L. (L. MIXTUS Krøyer)

The literature of pigmentary physiology contains, so far as my search has revealed, no account of chromatic responses in this species, though there are references (Fuchs, 1914) to other members of the genus. Younger specimens and usually the females (Lönnerberg and Gustafson, 1937) of this wrasse ("striped wrasse" or "red wrasse") are richly equipped with erythrophores but comparatively sparsely with melanophores, so much so that they are more or less old-rose in general tone. Both kinds of chromatophores occur fairly uniformly throughout the caudal fin; these are of moderate size and not very elaborate form. Smaller palish xanthophores are present also, but are irregularly distributed and in the tail fin are mostly grouped in blotchy patches.

Selecting the smallest specimens of the red phase available (15–20 cm. long), I tested their adaptive responses to colored bottoms by leaving groups of two each in the different colored bowls and then making macroscopic comparison and a quick microscopic inspection of the chromatophores in the tail fin.

An initial trial showed that running water was necessary. Kept an hour or more in the bowls without change of water, six fish were pale regardless of the ground color. The pigment concentration became obvious even in the iris, contrasting with its reddish, bright appearance in all healthy fish in running water, irrespective of the color of the bowl.

With water circulation provided, a distinct difference in shade appeared as response to dark and light bottom colors. Fish from the black bowl were darkest, those from the white, palest. As the range of difference was never wide, apparently not increased in periods longer than a day, the difference between the intermediately dark fish from the red bowl and those from the black or white was not striking, but it was verified on repetition and on interchange of pairs tested. On such interchange, the naked-eye effect induced by previous shorter or longer (even 2-day) stays over the given ground color reversed itself noticeably in three minutes and virtually completely in 15 minutes. These tests of themselves permit no conclusion regarding adaptation to hue as distinguished from shade. On the other hand, the caudal chromato-

phores, in attesting the reality of the response to shade, hinted at adaptation also as concerns hue. For example, all were practically in the concentrated state, on the average not quite punctate, in fish kept for two days over a white bottom. In those equally long over black, the melanin was fully dispersed and the red pigment also dispersed but not so widely; in those over red, the erythrophores showed more dispersal of pigment (though not extreme) than the melanophores, which were at most intermediate. Thus, the erythrophores and melanophores appear to respond differentially to a red as compared to a merely dark dish. Further tests with red vs. blue and red vs. yellow showed that the fish reacted differently to each of these three colors—becoming paler and less red over blue as against red, and paler and less red (in one case more yellowish) over yellow; and also darkening when shifted to red, but not when shifted to blue, from sand—but this study was not pursued far enough to verify decisively the apparent independence of erythrophore response to hue from melanophore response to shade.

The erythrophores resembled the melanophores in the reaction to handling and holding for microscopic inspection of the tail. In fish taken from white, dispersal of both pigments past the intermediate state occurred in half a minute or less. In fish taken from black, the contrary effect, general paling, ensued also very quickly. In no changes were the erythrophores conspicuously slower than the melanophores.

Innervated chromatophores of the body in some teleosts concentrate their pigment when chilled (Smith, 1928). This labrid on immersion in iced water quickly blanched, both melanophores and erythrophores over the body evidently exhibiting more intense concentration of pigment than observed under any other circumstances; but strong dispersal followed in 10–15 minutes (still iced), in fact became so complete in one fish, which then succumbed, as to obliterate the usually persistent whitish dorsal pattern spots.

In the case of another wrasse, *Crenilabrus*, with more striking changes of hue, von Frisch (1912) has shown that the rapidly reacting erythrophores have similar nervous control to that of the melanophores. Direct innervation of both types of chromatophores also in the red wrasse is suggested by the results of cutting a brace of caudal fin rays. At first the effects were mixed, the band distal to the cut turning dark red in only one of the five and seeming even blanched in one after 15 minutes. But after a day or two, by which time circulatory deficiencies were largely ameliorated, the band was distinct in two yellow-adapted, relatively pale fish, of four surviving, and became so in the other two red-adapted fish, when transfers to blue and to white induced pigment concentration in

the erythrophores and melanophores of all intact regions, those of the denervated sector remaining in the dispersed condition. After 15 days, mostly over a sand bottom but the last nine hours over blue, the band was still distinguishable, its erythrophores in a relatively dispersed state.

This evidence warrants no conclusion regarding the exact nature of the probably nervous control of melanophores and erythrophores in this wrasse. Yet no significant difference from the functioning of the probably doubly innervated melanophores of *Fundulus* has come to light.

PLEURONECTES PLATESSA L.

These notes are based on nearly 20 young specimens, 5.5–10 cm. long, of the European plaice. These fish had abundant small melanophores, about equally numerous xanthophores, of general distribution, and a few erythrophores, which, in the fins, were irregularly scattered. The layer of close-packed "iridocytes,"⁴ in which Meyer (1931) found the other chromatophores embedded, is represented even in the fins: the xanthophores are generally encircled by pale gray, opaque cells, active participants in pigmentary change, that further obscure the characteristically ill-defined xanthophores.

In a test of adaptive response to true color (denied by Schaefer, 1921, who admitted only change of shade in this species), two young plaice kept in a blue bowl for four days acquired a distinctly different coloration from two kept for the same time in a yellow bowl, the former becoming bluish gray (cf. Mast, 1916, on other flatfishes), the latter yellowish; left in white bowls, the two lots retained a noticeable difference for some hours, but lost all of it by the next day. Blanching and darkening responses to white and to black bowls were typically less slow—noticeable in half an hour—but varied according to previous history. For example, long-sustained pallor (white bowl) markedly slowed adaptive darkening (melanin dispersal). This fact implies the accumulation of a concentrating substance during white adaptation (Meyer, 1931).

The response to yellow kindled hope of obtaining evidence regarding cellular transmission of a dispersing neurohumor for comparison with that obtained from the xanthophores of *Fundulus* (Fries, 1942). But the results of cutting fin rays were uncertain, if not negative. Yellow bands due to pigment dispersal in the xanthophores were never strongly differentiated and, partly because of persistent incomplete concentration in the uncut sectors, were often not discernible at all, though they were found in several favorable instances, beginning three or four hours after the cut. These slowly reactive xanthophores seem likely to be at least

⁴ Again compare Odiorne (1933) *rê* guanophores and iridocytes.

predominantly under humoral control like that in *Phoxinus* (Giersburg, 1932).

The same experiments did, however, yield more significant data regarding the melanophores. Caudal bands (also dorsal-fin bands) from 2-ray or 3-ray cuts in pale or intermediate plaice regularly darkened, the melanophores showing dispersal, in less than an hour, excepting that in an instance of obvious circulatory stagnation the melanophores in the band assumed a fully concentrated state. The dark bands gradually faded in pale fish, full pigment concentration taking about two weeks in 3-ray bands (cf. Osborn, 1939).⁵ Faded bands redarkened more slowly than intact sectors under adaptation to black. A 19-day-old band showing this lagging dispersal (except at its proximal extremity, where nerve regeneration seemed to have begun) afforded a good demonstration also of the change progressing axipetally, as if dependent on invasions of a dispersing neurohumor—the melanophores next inside the band from the intact regions characterized by full dispersal came to show nearly full dispersal before those in the band axis changed appreciably from the concentrated state.

Out of 12 fish in which flanking and control secondary caudal bands were successfully established, five gave definite, and two others gave uncertain, indications of dispersal in the melanophores of the distal part of the faded primary band adjacent to the secondaries. A similar clear effect was elicited in just two out of the same number of equivalent operations on the dorsal fin. The mixed nature of these results recalls the case of the xanthophores of *Fundulus* (Fries, 1942).

Some sort of direct nervous control is well-attested in various other flatfishes, and Ballowitz (1893) has supplied morphological, and Schaefer (1921) physiological, evidence of it in the plaice. In so far as the spread of pigment dispersal from secondary bands into an adjoining primary band may be considered acceptable evidence of the existence of dispersing nerves and their neurohumor in *Fundulus*, one may conclude—without prejudice to the possibilities of supplementary influence by an adrenaline-like factor in the blood (Osborn, 1939), by the pituitary hormone (cf. E. Meyer, 1931, Osborn, 1939, H. H. Meyer, 1939a), or by a retinal substance reported by Meyer (1939b) to cause paling in the plaice—that my results with the plaice point to similar dual nervous control for the melanophores in this species.

⁵ As of interest in relation to the very long-maintained darkening of wide denervated bands observed by Osborn in *Pseudopleuronectes americanus*, it may be stated that 3-ray caudal bands in four *Pleuronectes flesus* (about 15 cm. long) failed to pale, except irregularly and along their margins, to match the pale innervated areas in the 26 days that I kept these flounders (over a gray-white bottom).

SUMMARY

Colored bowls evoked characteristic chromatophore responses in *Gobius minutus*, *Labrus ossifagus*, and *Pleuronectes platessa*. The responses, including those to blue and to yellow, effected differential adaptation of integumentary hue as well as shade, especially in the goby and the plaice. They confirmed that erythrophores, xanthophores, and melanophores may act independently, pigment dispersal in erythrophores and xanthophores depending on the color of the bottom while melanophores respond according to its darkness.

The changed chromatophore behavior resulting from fin cuts producing denervated bands indicated that the melanophores, erythrophores, and perhaps the xanthophores are innervated at least by concentrating fibers in the goby and that the erythrophores and melanophores are probably innervated in the wrasse.

Denervation experiments supplied evidence consistent with the hypothesis that dispersing as well as concentrating nerves and their neurohumors control the goby's melanophores and erythrophores and the plaice's melanophores. This evidence includes renewed pigment dispersal in the distal part of a faded, primary band where flanked by new, secondary bands (goby and plaice) and rapid dispersal in the not denervated melanophores and erythrophores when the fish were handled (goby).

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CONCERNING THE PIGMENTS OF THE TWO-SPOTTED OCTOPUS AND THE OPALESCENT SQUID¹

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The majority of fishes and invertebrate species of the sea exhibit integumentary colors due either to melanin pigments or, especially in the invertebrates, to carotenoids. Carotenoids are often also relatively concentrated in such tissues as the liver or digestive gland, and in eggs. Chromoproteins, flavines, purines, and certain more rare pigmentary compounds are discussed by numerous writers (e.g. Verne, 1926).

This paper concerns the pigments encountered in various tissues of two cephalopods of Pacific waters, namely the two-spotted octopus, *Paroctopus bimaculatus*, and the common squid, *Loligo opalescens*. Cephalopods studied by Lönnberg (1935) and the two species investigated by us have revealed a striking general absence of carotenoids from the integument and gonads and only traces in the eyes, but there are considerable quantities of these yellow, orange, or red pigments in the "liver"² of *Paroctopus*. Melanins characterize the ink of these mollusks, and are present also in the iris and skin. Octopods show relatively large quantities of melanin in their highly specialized chromatophores, while certain squids exhibit very little in the integument, the pigment there often appearing light brown or reddish instead of grey or deep brown as in the former group. We encountered, in the pericardial glands of the octopus, small amounts of yellow flavines as well as a red water-soluble pigment of unknown identity in the kidneys.

Lönnberg (op. cit.), making preliminary studies of the carotenoids of three cephalopods, reports lutein in the eyes of *Sepioloa scandica*, *Rossia macrosoma*, and *Eledone cirrosa*. He found no carotenoids in the eggs of *Rossia* or *Sepioloa*, and only possible traces in the testes of *Eledone*. Extracts of *Rossia* eggs, however, gave a strong color reaction with SbCl_3 , suggesting the possible presence of vitamin A or a similar com-

¹ Contributions from the Scripps Institution of Oceanography, New Series No. 154.

² The term as employed here refers to the large red-brown, grey or greenish gland communicating by a pair of ducts with the alimentary canal at the junction of stomach and intestine. Actually the "liver" and "pancreas" are incorporated together, without a very sharp division, in the single gland.

pound. Carotenoids were not detected in the skin of *Sepiola* or *Rossia*, but were reported in the mantle of *Eledone*. Especially notable was the presence of quantities of a carotenoid resembling lutein in the liver of *Eledone*. The livers of the other two species were not given special study.

Escher-Desrivières, Lederer and Verrier (1938) cite older observations of Kruckenberg on the purple pigments in retinae of certain cephalopods. Studying the retinal pigments of *Sepia officinalis*, *Octopus vulgaris*, and *Eledone moschata*, these authors found certain chemical and spectroscopic similarities between the cephalopod eye-pigments and those of classical types. They found, however, that the cephalopod retinal purple was stable in light, and they failed to find A vitamins in the material. More recent investigations by Wald (1941) have demonstrated vitamin A and retinene in the retina of the squid *Loligo pealii*, but no traces of these or other carotenoids in other squid tissues. According to Wald, vertebrates and invertebrates alike use vitamins A in the eye. Failure by other investigators to identify these carotenoids in the eyes of squids and other invertebrates is attributed by Wald to the low capacity of such forms for storing these substances. Wald points out that the quantity of vitamin A remains constant in the squid's eye in light and darkness, and that it may not participate directly in the visual processes. He claims that the deep purple pigment of the squid retina, sometimes referred to as visual purple, is really "a photostable, alkali soluble probably melanoid pigment," acting as a light-screen for the retinal cells, but probably playing no role in vision proper.

MATERIALS AND METHODS

Specimens of *Paroctopus bimaculatus* were taken freshly from the sea and killed by immersing in tap water just preceding analysis. Small specimens (about 6 inches in length) of the squid, *Loligo opalescens*, were bought from wholesale fish-dealers in San Diego. The animals showed no signs of decomposition, having been stored on ice since capture a few days earlier.

Brown or black melanin pigments were detected in the various tissues merely by visual inspection.

Flavines were characterized by their solubility in aqueous solutions, insolubility in organic solvents such as petroleum ether, bleaching of the yellow color by the addition of sodium hydrosulfite, reversible on shaking in air, and light absorption in the violet end of the spectrum near 417 μ .

Carotenoids were extracted, adsorbed on chromatographic columns,

and given chemical and spectroscopic study in accordance with standard methods described elsewhere. (Fox and Pantin, 1941; Scheer, 1940; Fox and Scheer, 1941.)

RESULTS

A preliminary survey, summarized in Table I, shows the distribution of certain of the pigments among various tissues of the two species, and reveals a few arresting facts. In *Paroctopus* we found no sexual dif-

TABLE I
Distribution of Pigments in the Tissues of *Paroctopus bimaculatus* and
Loligo opalescens

Species	Tissue	Weight, grams	Carot-enoids	Fla-vines	Melanins
<i>Paroctopus bimaculatus</i> ; two specimens, male (742 gm.) and female (778 gm.).	Salivary glands	♂ 3.8	—	—	?
		♀ 3.9	—	—	?
	Crop, stomach and intestine	♂ 4.2	traces	—	—
		♀ 6.4	traces	—	—
	Gills	♂ 6.4	traces	—	—
		♀ 9.1	traces	—	—
	Liver	♂ 13.8	+++	—	—
		♀ 20.1	+++	—	—
	Ink	1 to 2	+	—	+++
	Eyes	♂ 2.1	traces	—	+
		♀ 2.7	traces	—	+
	Gonads	♂ 13.2	traces	—	—
		♀ 2.3	—	—	—
	Pericardial glands	♂ 2.5	—	+	—
		♀ 2.4	—	+	—
	Kidneys*	♂ 1.3	—	—	—
		♀ 1.2	—	—	—
<i>Loligo opalescens</i> 7 ♂, 5 ♀	Heart	♂ 1.3	—	—	—
		♀ 1.3	—	—	—
	Muscle and skin†	♂ 640	—	?	} skin +
		♀ 709	—	?	} muscle —
	Eggs‡	33.8	traces‡	?	—
	Gills		—	—	—
	Digestive tract		—	—	—
	Ink and sac	2.8 (from 12 animals)	trace?	—	+++
	Eyes	16.5 (from 12 animals)	(+)	?	+
	Accessory nidimental gland	0.9 (from 12 animals)	traces	—	—
	Rest of body (including gonads)		traces?		traces

* The organs, purple in color, yielded quantities of a water-soluble, blood-red pigment discussed in text.

† Acetone extracts of muscle + skin gradually turned a pink-orange color, as did the ground tissue itself. This was not due to either carotenoids or flavines. The pigment was unchanged by acids or salts, but turned yellow in base, and its acetone solution gradually bleached in air.

‡ Laid by another female. Traces of yellow pigment soluble in petroleum ether or CHCl_3 , gave pink to mauve color in CHCl_3 with added SbCl_5 .

ferences in pigmentation. Here, as in *Loligo*, neither the mature testis nor the ovary or deposited eggs showed more than possible traces of carotenoids. The same was true in gills, digestive tract (minus liver) and eyes, while in the other tissues these pigments were absent. Carotenoids were yielded in considerable quantities by the relatively large liver, however, and were present in appreciable amounts in the ink stored in its sac, attached to the liver but not in direct communication with it. Flavines

were extracted only from the yellowish tripartite pericardial glands (of excretory function), while melanins characterized the ink, iris and skin.

The kidneys of *Paroctopus* were deep purple-red in color and when triturated yielded a blood-red water-soluble pigment. This pigment is insoluble in acetone or alcohol, being reversibly precipitated from water by these solvents. It contains iron and possesses reducing properties. Study of this pigment is being continued, and a more complete account of it will be published later.

Loligo was found to exhibit but little pigment of any kind in most of its tissues. Neither of the mature sexes yielded any pigments in the gonads. Flavines were not detected with certainty, and carotenoids were present in only very slight quantities in the eyes, while the other tissues yielded none or only suspected traces. While the body integument contained very little melanin this pigment was present in both iris and ink in considerable quantities.

Carotenoids in the Liver and in the Ink of the Octopus

The carotenoids were classified according to (1) their behavior in the partition test, both before and after hydrolytic treatment with alcoholic potassium hydroxide: carotenes and xanthophyll esters remain in the upper, or epiphasic layer of petroleum ether, while free xanthophylls migrate to the lower or hypophasic layer of 90 per cent methanol; following hydrolysis, carotenes remain unchanged, xanthophyll esters from the epiphase are split to give free xanthophylls which behave accordingly, while free carotenoid acids are now removed as potassium salts; (2) carotenes were separable one from the other by adsorption on Tswett chromatographic columns of $\text{Ca}(\text{OH})_2$, while xanthophylls were resolved into individual pigments on similar columns of CaCO_3 ; (3) finally, absorption spectra of the separate pigments were determined with a Hartridge Reversion Spectroscope or with a Bausch and Lomb Spectrophotometer. Carbon disulfide was employed as solvent in all of the carotenoid observations.

Liver

Carotenes, represented by from two to five colored zones on the chromatogram of the hydrolyzed epiphase, usually showed a fairly prominent red or orange zone near the top, and a yellow zone nearer the bottom of the column. One of the more prominent upper zones gave absorption maxima agreeing closely with those of β -carotene, i.e., $519.5\text{ m}\mu$ and $484.4\text{ m}\mu$ (Hartridge) and 510 to $512.5\text{ m}\mu$ and 482.5 to $485\text{ m}\mu$ (B. and

L. spectrophotometer). Another prominent carotene-like pigment appeared above the β -carotene zone, with absorption maxima at from 505 to 507.5 $m\mu$ and 480 $m\mu$. At times the shorter wave-length maximum had higher values of 484 to 486 $m\mu$. We did not identify this pigment with any known carotene.

Xanthophylls, present both free and as esters, were represented by considerable quantities of lutein (505 to 508 μ and 474.5 to 479 $m\mu$ by Hartridge; 505 to 510 $m\mu$ and 477.5 $m\mu$ by spectrophotometer), and by lesser amounts of other pigments of the taraxanthin or eloxanthin range of spectra (500 to 505 $m\mu$ and 469 to 473 $m\mu$); occasionally a third type was encountered, below the other two on the chromatogram, with absorption maxima not unlike those of antheraxanthin or petaloxanthin, i.e., 512 $m\mu$ and 481 $m\mu$ (cf. Strain, 1938).

Acidic carotenoids, appearing only after hydrolytic treatment, occurred in both the epiphase and hypophase.³ They always presented a single, broad and asymmetrical maximum, difficult to determine with precision in the Hartridge instrument, but giving thereby values from 501 to 507 $m\mu$, and, with the spectrophotometer, a broad hump between the values of 500 to 507 $m\mu$, with a slight maximum at about 503 $m\mu$.

Ink

Carotenes were absent.

Xanthophylls, both free and esterified, were found in smaller quantities than in liver tissue. Hydrolytic treatment did not permit subsequent recovery of sufficient quantities for critical spectroscopic study. Inspection of the fresh ink extract, however, revealed, in one instance, absorption maxima similar to those of lutein (510 $m\mu$ and 475 $m\mu$). The epiphasic pigments of the ink carotenoids dissolved in carbon disulphide gave an orange solution of esters with maxima at 502.6 $m\mu$ and 472 $m\mu$ (cf. eloxanthin), while the hypophasic pigments in carbon disulfide failed to yield sharp maxima. Hydrolysis of the epiphase yielded free xanthophylls accompanied by the familiar carotenoid acid. The hypophase yielded, on hydrolytic treatment, an acidic carotenoid, sometimes but not always accompanied by a non-acidic xanthophyll.

In Table II are presented data concerning the relative mass of liver tissue and the range of concentrations of carotenoids encountered in liver and ink. Since the preponderant carotenoid of both liver and ink exhibited, in chemical, adsorptive, and spectroscopic behavior, properties agreeing closely with those of lutein, the concentrations of mixed carotenoids were measured, for purposes of comparison, in "lutein equivalents" with a Bausch and Lomb visual spectrophotometer.

³ Always hypophasic after hydrolysis of the extract.

The combined weight of liver and ink is seen to vary between 1.86 per cent and 3.29 per cent of the total body weight, with a mean value of about 2.7 per cent. We were rarely able to recover more than one or two grams of the ink, which represented from 5 to 11 per cent of the combined weight of liver, ink-sac and ink.

The concentration of carotenoids (in lutein equivalents) in liver tissues ranged between 1.2 and 8.3 mg. per 100 gm. with a mean value of about 3.5, in freshly excised tissue from normal animals, i.e., excluding the relatively low value of 0.8 given by the autolyzed liver of animal No. 7. The ink of freshly caught specimens yielded appreciable amounts of

TABLE II
Concentrations of Carotenoids in Liver and Ink

Animal (sex)	Weight, grams	Liver + ink weight, grams	Liver and ink % of total body weight	Total carotenoids (as lutein) mg./100 g.
1 ♂	742	13.8	1.86	2.4
2 ♀	778	20.1	2.58	2.1
3 ♂	716	20.6 (liver) 2.3 (ink)	3.19	1.2 0.70
4 ♂	721	18.9 (liver) 1.1 (ink)	2.77	3.9 0.55
5 ♂ (starved)	820	25.4 (liver) 1.6 (ink)	3.29	 ¹
6 ♂ (immature)	493	12.7	2.57	8.3 ²
7 ♂	623	15.1	2.42	0.82 ³

¹ Very little of the pigment in this specimen was carotenoid, having evidently been altered by starvation.

² Quantity of carotenoids unchanged by incubating toluene-preserved macerated liver at its natural pH (5.42) for 44 hours at 37° to 40° C.

³ Liver + ink autolyzed by placing in closed flask in presence of a few drops of CHCl₃ for 16 days at room temperature.

carotenoids showing values of from 0.55 to 0.7 mg. per 100 gm. (mean value from two determinations 0.62 mg. per 100 gram) while the ink of one specimen (No. 5) which had been starved for two or three weeks was found to be completely lacking in carotenoids.

DISCUSSION

Regarding the chief role of the liver-pancreas in cephalopods, Jordan (1929) states that it possesses only a secretory function, elaborating digestive enzymes which are passed into the gut during feeding.

In its lack of absorptive function, and its secretion of extracellular enzymes, the liver of cephalopods differs markedly from that of lamelli-branch mollusks (see Yonge, 1931). The California mussel, a typical

phytoplankton feeder, passes great amounts of minute plant plastids into its digestive diverticulum, or "liver," whence certain cells engulf and absorb food, passing the undigested residues back into the digestive tract (Fox et al., 1936; Scheer, 1941, and unpublished observations on voiding of feces during early starvation). It would appear obvious that the liver of the mussel receives its rich supply of carotenoid pigments in a direct way from the plant material taken in. The liver of the octopus, on the other hand, probably receives its supply of carotenoids through the blood stream.

Very little seems to be known of the physiology of ink secretion in cephalopods, or of the biochemistry of the ink itself, beyond its rich melanin content. Its carotenoid content provokes much thought. We are aware of the loss of carotenoids from ectoderm in various ways, such as the sloughing of skin, the growth of feathers in certain birds, the discharge of ear-wax in cattle, and the presence of such pigments in the cores of solid materials secreted from the femoral pits of iguanas (unpublished data). Save for the storage of carotenoids in egg-yolk of many oviparous vertebrates and invertebrates, and the secretion of such pigments in the milk fat from mammary glands, the discharge of carotenoids from an internal structure to the outside would appear to be rather unique.

Carotenoids of *Paroctopus* disappeared from both liver and ink in a starved specimen, yet were reduced in amount only very slowly on autolysis of the liver at room temperature, or by incubation of the ground tissue at 37° C.

Clearly, a thorough investigation of the physiological role of the liver is called for, likewise a biochemical study of the ink and its relationship to the metabolism of the animal as a whole, with particular reference to the liver.

SUMMARY

The kinds and tissue-distribution of various pigments in the cephalopods *Paroctopus bimaculatus* and *Loligo opalescens* are described and discussed. Melanins, stored in quantity in eyes and ink of both species, are far more plentiful in the integument of the octopus than in that of the squid.

Flavines were detected with certainty only in the pericardial (excretory) glands of the octopus. This species also yielded, from its kidneys, a water-soluble, iron-containing red pigment with reducing properties.

Carotenoids were present only in traces in any of the organs of the squid. This was likewise true of the octopus, save in the liver-pancreas of this species, which contained a variety of carotenoids in amounts of

3.5 mg. (lutein equivalents) per 100 grams of moist tissue (average value). Ink from the sac adjacent to the liver contained 0.55 to 0.70 mg. lutein equivalents per 100 grams.

In the liver of *Paroctopus*, free and esterified xanthophylls, predominantly of the lutein class, were accompanied by smaller amounts of other xanthophylls, β -carotene and an additional unfamiliar carotene, and a unique carotenoid acid, appearing on hydrolysis, not identical spectroscopically with astacene or with the metridene of Fox and Pantin.

The ink of this species yielded no carotenes, but free and esterified xanthophylls and (on hydrolysis) a carotenoid acid similar to respective compounds recovered from the liver. Whilst starvation brought about the gradual disappearance of carotenoids from liver and ink, autolysis of the liver or incubation of its ground tissues resulted in only slow loss of the pigments.

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STUDIES ON DEROPRISTIS INFLATA (MOLIN), ITS LIFE HISTORY AND AFFINITIES TO TREMATODES OF THE FAMILY ACANTHOCOLPIDAE

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INTRODUCTION

During the summer of 1939, three undescribed species of cercariae, one of which was a modified trichocercous larva, were found in the marine snail, *Bittium alternatum*, from Waquoit Bay, Cape Cod, Massachusetts. This trichocercous cercaria penetrated and encysted in *Nereis virens* and this annelid, when collected in the vicinity of infected snails, was found to be naturally infected. Of the trematodes that had been found in an extended survey of fish parasites in the vicinity, only two species could be regarded as possibly the adult of the cercaria in question; these were *Deropristis inflata*, belonging to the family Acanthocolpidae, and *Homalometron pallidum*, a species allocated to the subfamily Anallocreadiinae, family Allocreadiidae. Of the two, *H. pallidum* was at first believed most likely to be the adult of the cercaria from *B. alternatum*, since Hopkins (1937) had described remarkably similar cercariae for *Anallocreadium* and *Microcreadium*, both anallocreadiine genera closely related to *Homalometron*. This belief was supported by a comparison of the present cercaria with the adult of *H. pallidum* which showed that they agreed in possessing eye-spot pigment, a short, sac-shaped excretory vesicle, ciliated main excretory tubules reaching only to the ventral sucker, and similar branching of secondary tubules.

Each new life history study provides increasing evidence that trematodes properly allocated to the same family have life cycles involving similar embryological and larval stages and usually intermediate hosts that are closely related. It seemed unlikely, therefore, that *D. inflata*, a member of the family Acanthocolpidae, would have a trichocercous cercaria that encysted in annelids, since Martin (1939) had shown that another acanthocolpid, *Stephanostomum tenue*, has an ophthalmoxiphidio-cercaria which encysts in fishes. Furthermore, metacercariae of at least

¹ This work was assisted by a grant-in-aid to the junior author from the Society of the Sigma Xi.

two other genera in the family are known to occur in fishes. Although Carrère (1937, 1938) had identified a metacercaria from *Nereis dumerilli* as that of *D. inflata*, his observations did not seem at first to be significant for the present study since a number of cercariae encyst in species of *Nereis* and the most variable phase of the trematode cycle is that enabling the parasite to reach the definitive host. Feeding experiments, however, have demonstrated conclusively that the cercaria from *B. alternatum* is that of *D. inflata* and that the family Acanthocolpidae, as constituted at present, is not a natural group of closely related trematodes.

Although numerous species have been described and allocated to certain acanthocolpid genera, particularly *Stephanostomum* (here regarded as taking precedence over *Stephanochasmus* in accordance with the recommendation in Article 36 of the Rules of Nomenclature), a critical analysis of the family has been prevented by a lack of information concerning the type genus *Acanthocolpus*, and particularly the excretory system and life histories of acanthocolpids. The imperfectly described *Acanthocolpus liodorus* was the only known representative of the type genus until Srivastava (1939) described three new species of *Acanthocolpus* from Indian marine fishes. A better understanding of the type genus, and knowledge of the life histories and excretory systems of *Stephanostomum tenue* and *Deropristis inflata*, afford an opportunity to analyze the interrelationships of the acanthocolpid trematodes and their affinities to other groups.

HISTORICAL REVIEW

Ward (1938) has reviewed the family Acanthocolpidae and the genus *Deropristis*, supplementing previous descriptions of the two known species, *D. inflata* (Molin) and *D. hispida* (Albildgaard). References concerning these species may be obtained from Ward's paper and need not be repeated here. A brief review of the family is desirable as a basis for discussion later.

The first attempt to group the trematodes at present allocated to the family Acanthocolpidae was made by Lühe (1906) who proposed the subfamily Acanthocolpinae (family unmentioned) to contain the genera *Acanthocolpus*, *Stephanochasmus* (syn. *Stephanostomum*), and *Deropristis*, and the species *Distomum semiarmatum* Molin. Later, Lühe (1909) established the family Acanthocolpidae, apparently to contain all these forms although he mentioned only *Deropristis inflata*, *D. hispida*, and *Distomum semiarmatum*; these species occur in migratory fishes and Lühe was concerned with only freshwater forms. He did not propose a subfamily Acanthocolpinae at that time, as assumed by some authors,

but stated that the various genera apparently belonged to several subfamilies. Odlner (1911), evidently disregarding Lühe's proposal, stated that *Stephanochasmus*, *Dihemistephanus*, *Acanthopsolus*, and *Distomum osculatum* Looss constituted a distinct family but did not propose a name for it. Nicoll (1913) erected the genus *Lepidauchen* which seemed to him to be an intermediate form combining the characters of the genera *Lepocreadium* and *Stephanochasmus*. He later (1915) placed *Lepidauchen* in the Lepocreadiinae, *Stephanochasmus* and *Acanthopsolus* in the Stephanochasminae, all of the family Allocreadiidae, and *Deropristis* in a list of forms of uncertain position. Poche (1925) criticized Nicoll's allocation of *Stephanochasmus* and *Acanthopsolus* to the family Allocreadiidae, removing these genera to the family Acanthocolpidae, to which he added *Dihemistephanus*, *Acanthopsolus*, and the new genus *Tormopsolus*. He also followed Pratt (1916) in reducing *Lechradena* to synonymy with *Stephanochasmus* and Odlner (1911) in the opinion that *Neophasis* is synonymous with *Acanthopsolus*. To include a new genus, *Echinostephanus*, Yamaguti (1934) redefined the subfamily Stephanochasminae, placing it in the family Acanthocolpidae. Ward (1938) denied the validity of both the genus *Echinostephanus* and the subfamily Stephanochasminae, but proposed a new subfamily, Acanthopsolinae, for the single genus *Acanthopsolus*.

MATERIALS AND METHODS

During the summers 1939-41, several thousand *Bittium alternatum* were examined for larval trematodes. The snails were collected from Waquoit Bay at Menauhant, Massachusetts, and the infected specimens were separated by isolation to provide an abundant supply of cercariae. To obtain metacercariae of known age, a specimen of *Nereis* was placed in a large crystallizing dish and exposed to all the cercariae that emerged from a number of infected snails during a 24-hour period. After exposure, each annelid was kept either in a finger bowl half filled with sand, covered with cheesecloth, and placed in running sea water, or in a dish of water which was aerated without changing. Various species of fish for feeding experiments were isolated in the laboratory for at least three weeks before use. The experimental animals were killed and examined from one to seven days after being fed infected annelids. If, kept for longer periods, the worms either failed to establish themselves in unsuitable hosts such as *Fundulus* and flounders, or the eel, the natural host, they developed until they could not be distinguished with certainty from worms that might have been present when the fish was collected.

Throughout the study, living material was used extensively although

measurements were taken mostly from recently killed or fixed and stained specimens. Since the living cercaria is extremely active and difficult to measure without considerable coverglass pressure, measurements were taken mostly from larvae that were killed by placing them on a slide in a large drop of water, covering and holding over a flame for a moment. When treated in this manner, practically every cercaria died in a moderately extended, straightened position, and the body did not become opaque as when killed with the usual fixatives.

Neutral red and Nile blue sulfate were used supravitaly and permanent whole mounts were stained with paracarmine. All measurements are in millimeters and all figures, except those indicated as free-hand drawings, were made with the aid of a camera lucida.

We wish to express our thanks to Dr. H. W. Manter for the privilege of examining material of *Dihemistephanus brachyderus* and to Professor H. W. Stunkard for helpful suggestions and criticism during the preparation of the manuscript.

OBSERVATIONS

Proof of the Life History of Deropristis inflata

Since at first we suspected that the cercaria was the larva of *Homalometron pallidum*, both naturally and experimentally infected annelids were fed to four *Fundulus heteroclitus*, a species which serves as the natural host of that trematode. These fish were examined five days later and found to be uninfected. Next, a large *Nereis*, heavily infected with 14-day metacercariae, was fed to a summer flounder, *Paralichthys dentatus*, which had been isolated for four weeks and had begun to take food. When killed and examined the following day, several hundred recently excysted worms were recovered from the intestine. These worms were all the same size and showed the conspicuous spines of the dorsal hump and lateral forebody characteristic of *Deropristis*. This observation was made too late to conduct further experiments until the following summer (1941) when the study was continued and the feeding of experimentally infected annelids to eels yielded large numbers of immature *Deropristis*. Control fish were either negative for *D. inflata* or harbored only specimens in an advanced stage of development. A careful study of the metacercaria revealed that the peculiar spination of *Deropristis* was just beginning to differentiate, the dorsal and lateral spines being distinctly larger than adjacent body spines.

Finally, a careful comparison of the excretory system of the cercaria and that of the young adult in both natural and experimental infections showed complete agreement. Thus the experimental results

and morphological evidence afford conclusive proof of the life history of *D. inflata*.

Description of stages in the life history of Deropristis inflata
Adult (Figs. 1-5)

The common eel, *Anguilla rostrata*, serves as the natural definitive host of *D. inflata* in the Woods Hole region. Practically every eel examined, except those that had been in captivity for several weeks, was found to be infected. The worms occurred throughout the intestine, being more numerous toward its posterior end.

The fairly complete accounts given by Odhner (1902) and Linton (1940) and supplementary observations by Manter (1926) and Ward (1938) make it unnecessary to give a complete redescription of *D. inflata*. Measurements of our specimens are well within the limits given in other descriptions. General morphological features are shown in Figs. 1-4. Since extensive observations have been made on living specimens, it is desirable to supplement existing descriptions of body spination and the reproductive and excretory systems.

EXPLANATION OF PLATE I

(All figures concern *Deropristis inflata*)

- FIG. 1. Mature adult, ventral aspect.
 FIG. 2. Spines of (A) metraterm and (B) cirrus (free-hand).
 FIG. 3. Adult. Dorsal aspect of anterior end showing details of spination (many small spines omitted).
 FIG. 4. Adult. Lateral view showing details of spination and terminal genitalia (many small spines omitted).
 FIG. 5. Female reproductive system, dorsal view (free-hand).
 FIG. 6. Embryonated egg from terminal portion of uterus.

ABBREVIATIONS

- | | |
|--------------------------------------|--|
| <i>CI</i> , cirrus. | <i>OS</i> , oral sucker. |
| <i>CS</i> , cirrus sac. | <i>OT</i> , oötype. |
| <i>CV</i> , common vitelline duct. | <i>OV</i> , ovary. |
| <i>EG</i> , egg. | <i>PH</i> , pharynx. |
| <i>EP</i> , excretory pore. | <i>PR</i> , prostate cells. |
| <i>ES</i> , esophagus. | <i>RV</i> , right vas efferens. |
| <i>EV</i> , excretory vesicle. | <i>SC</i> , cervical spines. |
| <i>EY</i> , eye-spot pigment. | <i>SD</i> , dorsal hump spines. |
| <i>GA</i> , genital atrium or sinus. | <i>SR</i> , seminal receptacle. |
| <i>GL</i> , cervical gland. | <i>SV</i> , seminal vesicle. |
| <i>GP</i> , genital pore. | <i>TE</i> , testes. |
| <i>IN</i> , intestine. | <i>TV</i> , transverse vitelline duct. |
| <i>LC</i> , Laurer's canal. | <i>UT</i> , uterus. |
| <i>LV</i> , left vas efferens. | <i>VD</i> , vas deferens. |
| <i>ME</i> , metraterm. | <i>VI</i> , vitellaria. |
| <i>MG</i> , Mehlis' gland. | <i>VR</i> , vitelline reservoir. |
| <i>OD</i> , oviduct. | <i>VS</i> , ventral sucker. |

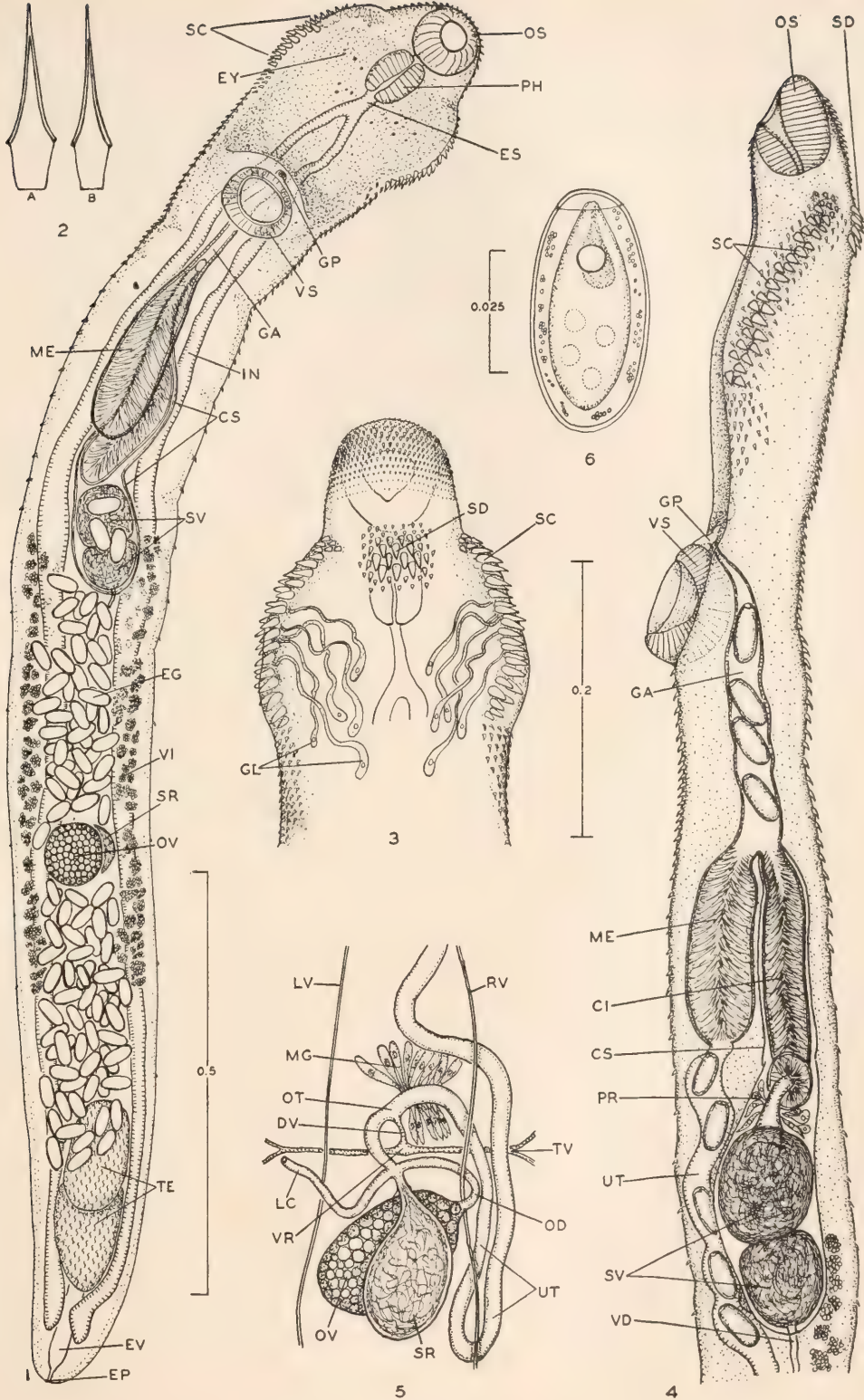


PLATE I

Although spination of the body has been described rather fully, certain features have apparently been overlooked. The first three rows of spines behind the ventral lip of the oral sucker are slightly larger than those posterior to this level and the concave ventral surface of the body is devoid of spines from about the level of the cervical expansions to the ventral sucker. The large spines of the dorsal hump (Fig. 3) and probably also those of the cervical expansions (Fig. 4) develop at first as members of rows of spines quincuncially arranged. The number of enlarged spines on the dorsal hump is variable, but in very young worms there are usually ten in transverse rows of three, four and three. Adjacent spines are more or less enlarged in mature worms and the large spines become very prominent, more or less masking the original quincuncial pattern. They may become worn with age or even lost, giving the dorsal hump a ragged appearance. In living specimens, numerous conspicuous glands in the forebody are seen to open among the prominent spines of the cervical expansions. The ducts of a few such glands also open on the ventral surface of the forebody.

Living *D. inflata* is extremely favorable material for observing the reproductive system, the details of which can be observed completely in almost every specimen. The oviduct (Fig. 5) extends from the right side of the ovary for a short distance and then turns dorsally and to the left where its junction with the duct of the seminal receptacle, Laurer's canal, and the oötype forms a cross. From this junction, Laurer's canal extends to the left, opening at the dorsal surface considerably to the left of the median line. The oötype extends forward a short distance and then turns to the right, immediately receiving the duct of the vitelline reservoir. Just beyond this point, the oötype is surrounded by a moderately developed Mehlis' gland. From the oötype, the uterus extends posteriorly to the right of the ovary and returns on the same side. In young worms, this posterior loop does not extend behind the ovary while in large, mature specimens, it may form convolutions between the female complex and the anterior testis. In front of the ovary, the uterus extends anteriorly as a moderately sinuous tube and joins the prominent metraterm with its conspicuous lining of spines. The vitelline follicles are diffuse and confined to the region between the cirrus and the anterior testis. The transverse vitelline ducts, which unite to form the vitelline reservoir, are ventral to the remainder of the female complex.

In large worms, the eggs are fairly numerous, and in fresh material measure from 0.042 to 0.052 mm. long and 0.024 to 0.026 mm. wide. They are fairly thick-shelled and operculate; older ones are brown in color and contain active embryos, but eggs teased from worms and kept for ten days in sea water failed to hatch.

The testes (Fig. 1) lie near the posterior end of the body, one behind the other or slightly overlapping. The vas efferens of the anterior testis is to the left of the median line while that of the posterior testis is to the right. The left vas efferens passes between Laurer's canal and the vitelline duct of that side, while the right runs through the loop of the oviduct. Anterior to the female complex, the vasa efferentia unite to form the vas deferens which is about as long as the seminal vesicle and slightly enlarged posteriorly. The cirrus sac is longer than the metraterm and contains the seminal vesicle, pars prostatica, and prominent, spined cirrus. A short distance posterior to the ventral sucker, the metraterm and cirrus sac join the slender, tubular genital sinus. The largest spines of the metraterm and cirrus (Fig. 2) are about 0.04 mm. long, and of similar structure, but slightly different in shape, those of the metraterm being a little wider. Each spine consists of an acute, refractile point and a very delicate, hyaline medullary and basal portion, and is very different from the body spines in structure, as noted by Olsson (1867) and Ward (1938). In one aberrant specimen, spines similar to those of the metraterm were observed in the genital sinus.

The excretory system (Fig. 11) has been completely traced in young worms obtained from both naturally and experimentally infected eels. The excretory pore is at the posterior end of the body. The vesicle is sac-shaped and extends anteriorly as far as the anterior testis. The main excretory tubules are ciliated throughout their length and extend from the anterolateral edges of the vesicle almost to the ventral sucker where each receives an anterior and a posterior collecting tubule. Each of these tubules serves three groups of flame cells but the disposition of secondary tubules and numbers of flame cells in the groups are not the same for anterior and posterior halves of the system. These differences may be noted in Fig. 11. There may be five, six, or even more flame cells in the posterior-most group on each side, depending on the size of the worm. One flame cell of this group is larger than the others and has a more conspicuous capillary which joins the collecting tubule separately from and anterior to the capillaries of the remaining flame cells of the posterior group. There is also a marked lack of dichotomy in the arrangement of the capillaries in other flame cell groups. The excretory formula of fairly young adults is $2[(3 + 5 + 7) + (4 + 4 + 6)] = 58$ flame cells. There is an increase in the number of flame cells during post-cercarial development but only in the groups associated with the posterior collecting tubules.

The Cercaria (Figs. 7-8)

Specific Diagnosis.—Distome modified trichocercous type. Body pyriform in shape with maximum width anterior to ventral sucker; spi-

nose anterior to eye-spots; extended length over 0.4, contracted less than 0.13. Tail extended 0.33, contracted 0.19. Measurements of 10 cercariae killed by heat: body length 0.21–.28 (average 0.25); maximum width 0.08–.1 (0.086); length of tail 0.2–.23 (0.21), width near base 0.026–.03 (0.028); oral sucker diameter 0.04–.045 (0.043); ventral sucker 0.043–.045 (0.044), situated in posterior half of body; average length of prepharynx 0.023, pharynx 0.017; eye-spots 0.011 wide. Tail typically with a ventral fin-fold and 6 pairs of ventrolateral tubercles, each with a long delicate “hair.” Prepharynx moderately long, pharynx present, intestinal ceca extending almost to posterior end of body. At least eight pairs of cephalic glands anterior to ventral sucker; numerous gland-like cells and refractile droplets throughout body. Genital primordium an undifferentiated mass posterior to ventral sucker. Excretory vesicle sac-shaped, thick-walled and with numerous concretions. Main excretory tubules ciliated. Flame cell formula $2[(3 + 5 + 7) + (3 + 3 + 5)] = 52$ flame cells. Develop in rediae in the branchial region and digestive gland of the molluscan host.

Host: Bittium alternatum (Say).

Locality—Waquoit Bay, Falmouth, Massachusetts, U. S. A.

Study of the excretory system of the cercaria in the light of observations on the adult stage necessitates correcting our preliminary abstract (Cable and Hunninen, 1940). The excretory formula of the cercaria, as included in the above specific diagnosis, is not in agreement with that given in the abstract. The flame cells and capillaries of the cercaria are extremely small and the presence of numerous glands and refractile droplets makes it very difficult to observe details of the excretory system.

EXPLANATION OF PLATE II

(All figures concern *Deropristis inflata*)

FIG. 7. Cercaria, ventral aspect.

FIG. 8. Cercaria, median sagittal (optical) section of anterior end showing papilla resembling stylet (free-hand).

FIG. 9. Redia, showing excretory system of one side (free-hand).

FIG. 10. Five-day metacercaria.

FIG. 11. Excretory system of young adult (free-hand).

ABBREVIATIONS

CG, cephalic glands.
CT, caudal tubercle.
EC, excretory concretion.
EP, excretory pore.
EV, excretory vesicle.
EY, eye-spot.
GD, cephalic gland ducts.
IN, intestine.

OS, oral sucker.
PG, genital primordium.
PH, pharynx.
PP, prepharynx.
PS, stylet-like papilla.
RD, refractile droplets.
VF, ventral fin-fold.
VS, ventral sucker.

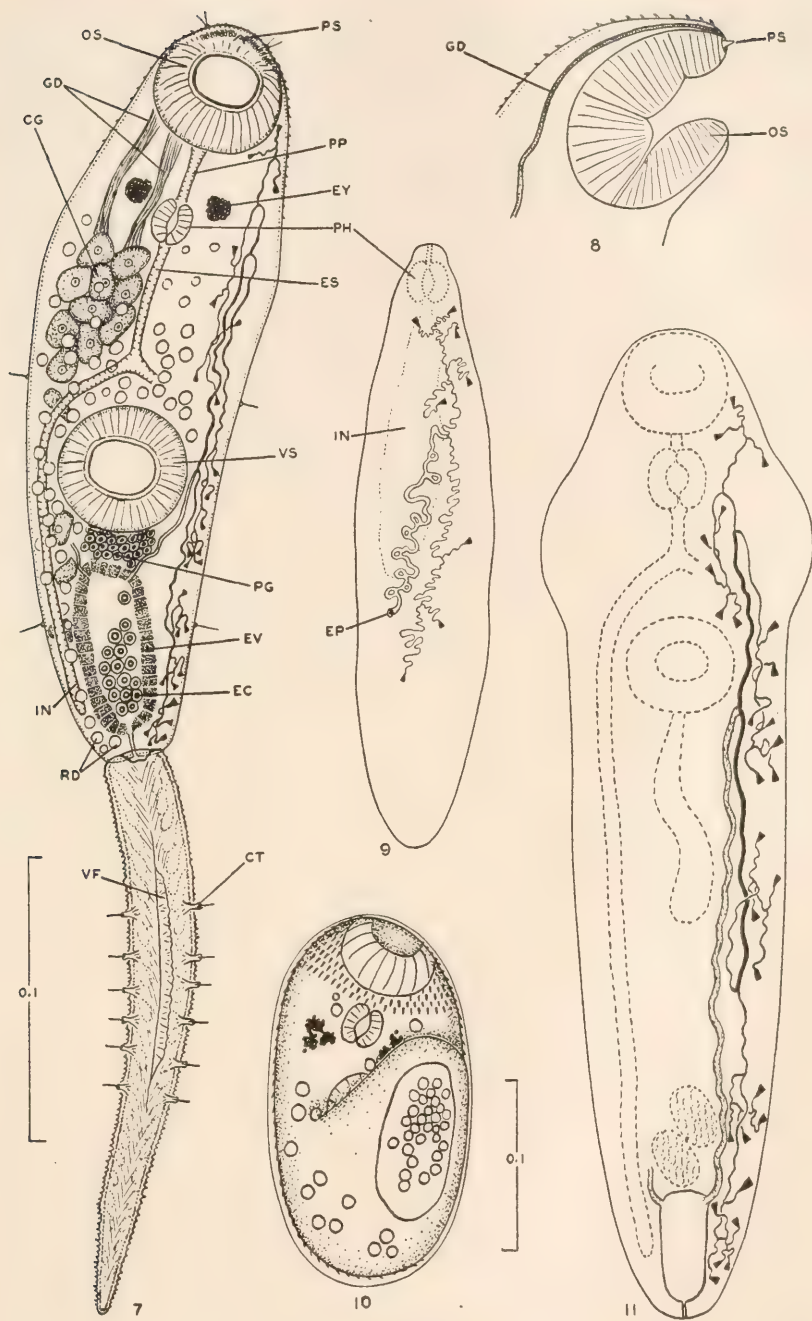


PLATE II

For several hours after emerging from the snail, the cercariae of *D. inflata* swim almost continuously, tail first, lashing in a figure eight, with the body flexed so that the anterior end is pointed in the direction of swimming. When dissected from the snail, they are photopositive but naturally emerging cercariae are decidedly photonegative and collect at the side of the dish farthest from the light. Precisely the same behavior has been described by Hopkins (1937) for the cercariae of *Anallocreadium* and *Microcreadium*. In one set of experiments with alternate illumination of opposite sides of a syracuse dish, it was observed that when the direction of the light was reversed, the cercariae would swim as a rather compact group across the dish in less than one minute. By illuminating both sides of the dish at once, the larvae scattered; swimming in a hesitant manner. In many cases, decaudation was noted after which the cercarial body remained alive for several hours.

The body of the cercaria is widest apparently at the level where the prominent cervical expansions will develop later. It tapers posteriorly and is devoid of general pigmentation. The eye-spots are conspicuous and contain a refractile body. When contracted the tail is almost as wide as the posterior end of the body and has finely crenated edges. The fin-fold is narrow and crinkled. Although the tail typically has six tubercles symmetrically arranged along each side, there is considerable variation in the number, size, and arrangement of these structures. Accessory tubercles may be present and one specimen was observed in which they were seen on only one side of the tail.

The dorsal lip of the oral sucker bears a blunt, papilla-like structure (Fig. 8) which is difficult to interpret. It is about 0.004 mm. long, conical, non-refractile, and set in what appears to be a slight depression. This structure was first regarded as possibly a rudimentary stylet. However, it is directed anteriorly rather than dorsally as in "allocreadiid" cercariae and has more the appearance of a papilla; the ducts of the cephalic glands do not converge toward it but instead open in a row along the dorsal lip of the oral sucker. We are therefore inclined to believe that the structure in question is not homologous with the stylet of other cercariae. Consequently, we can see no reason to postulate that the cercaria of *D. inflata* is an intermediate type between the ophthalmoxiphidiocercariae and trichocercous forms.

Of the described species of trichocercous larvae, the cercaria of *D. inflata* most closely resembles the cercariae of *Anallocreadium* and *Microcreadium* as described by Hopkins (1937). In fact, it was this resemblance that first led us to suspect that *Homalometron pallidum* might be the adult stage of the larva we were studying. A comparison of the cercariae of *Anallocreadium*, *Microcreadium*, *Deropristis*, and *Stepha-*

nostomum will be given in connection with an analysis of the family Acanthocolpidae in the discussion.

The Redia (Fig. 9)

Two redial generations were observed. The simple elongate daughter redia attains a length of over 1 mm. In living specimens, the pharynx measures 0.045 mm. in diameter and is followed by a short, expanded intestine with orange-yellow contents. There is a birth pore near the pharynx. Cercariae reach apparently complete development in the redia which may contain as many as 14 active larvae. In addition to cercariae, even the largest redia contains a group of germ cells situated at the posterior end of the brood cavity.

There is a striking homology between the excretory system (Fig. 9) of the daughter redia and that of the cercaria. Not only are the main excretory tubules ciliated in both stages, but the branching of the tubules and the number of flame cell groups are essentially the same, although with the exception of the anterior-most, the flame cell groups of the cercaria and adult are represented in the redia by single flame cells. As compared with the adult, then, the daughter redia has the complete flame cell complement in only the anterior-most group while the cercaria has the adult complement in three groups or all those connected with the anterior collecting tubule. While we have not had favorable material for determining the excretory patterns of the miracidium and mother redia, the above observations afford convincing evidence of recapitulation in the development of the excretory system in the various life history stages.

The Metacercaria

When annelids were exposed to large numbers of cercariae, massive infections were obtained. The larvae encyst mostly in the axial body region of *Nereis*, although in heavy infections, they occur to a less extent in the parapodia. The cyst is oval in shape, and the primary cyst membrane is thin, and difficult to remove from the worms without injuring them. In older metacercariae, the cysts may become brown in color, particularly when localized in the parapodia. Ten living 5-day metacercariae (Fig. 10) measured 0.17–.23 mm. long and 0.11–.14 mm. wide. The metacercaria does not increase appreciably in size with age although a certain amount of development occurs. Five days after encystment, the sharp-pointed body spines, characteristic of the adult, are present well behind the ventral sucker and those which later become very prominent on the dorsal hump and at the sides of the forebody are just detectably larger than the remaining body spines. Also at this age, the

eye-spots begin to dissociate and the concretions of the excretory vesicle have more the appearance of oil droplets than in the cercaria.

DISCUSSION

The results of the present study and a review of the literature demonstrate certain fundamental differences between *Deropristis* and other genera heretofore allocated to the family Acanthocolpidae. A redefinition of the family will be helpful in summarizing these differences.

Family Acanthocolpidae Lühe, 1909, emend.

Oval or elongate distomes, body weakly muscular, oval to round in cross-section. Cuticle spinose, frequently with large spines associated with oral sucker. Remnants of cercarial eye-spots often present. Suckers relatively close together. Prepharynx usually long, pharynx well developed, esophagus short; intestinal ceca extend almost to posterior end of body, rarely joining the excretory vesicle or with diverticula. Excretory vesicle tubular, not reaching ventral sucker, and with a pair of main excretory tubules extending from its anterior end to cephalic region of body before receiving secondary tubules. Genital pore median or submedian, immediately anterior to ventral sucker or rarely opening within ventral sucker; genital atrium usually tubular, posterior portion sometimes with spines; cirrus sac present, metraterm and cirrus often with prominent spines. Testes two (rarely multiple), in posterior region of body, tandem or obliquely one in front of the other; ovary near or some distance in front of anterior testis; seminal receptacle lacking, uterine seminal receptacle frequently present; vitelline follicles numerous, reaching from level of cirrus sac to posterior end of body. Uterine coils preovarian and intercecal, not extensive; eggs relatively few, operculate and without filaments. Adults parasitic in intestine of marine fishes, metacercariae in fishes. Cercaria of ophthamoxiphidiocercous type, developing in simple rediae in marine gastropods. Type genus, *Acanthocolpus* Lühe, 1906. Includes also:

Stephanostomum Looss, 1899 (syn. *Stephanochasmus* Looss, 1900; *Lechradena* Linton, 1910).

Dihemistephanus Looss, 1901.

Neophasis Stafford, 1904 (syn. *Acanthopsolus* Odhner, 1905).

Tormopsolus Poche, 1925.

Echinostephanus Yamaguti, 1934.

Lepidauchen Nicoll, 1913.

Pleorchis Railliet, 1896 (syn. *Polyorchis* Stossich, 1888, preoccupied).

Pseudolepidapedon Yamaguti, 1938.

A careful comparison of the above genera indicates a sufficiently close interrelationship to preclude the necessity of subfamilies.

In contrast with the family Acanthocolpidae as set forth above, *Deropristis* has a modified trichocercous cercaria which encysts in annelids; the main excretory tubules barely reach the ventral sucker; there is a well developed seminal receptacle independent of Laurer's canal; vitelline follicles are scanty and restricted to the region between the cirrus sac and the anterior testis; the uterine coils extend into the space between the ovary and anterior testis; and the prepharynx is shorter than the esophagus. These differences fully justify the exclusion of the genus *Deropristis* from the Acanthocolpidae.

The above definition of the family excludes also the species described by Little (1930) as *Dihemistephanus sturionis*; it is very doubtful whether this form belongs in either the genus *Dihemistephanus* or the family Acanthocolpidae, since Laurer's canal is enlarged to form a seminal receptacle, the vitelline follicles as figured appear scanty and do not extend to the extreme posterior end of the body while the uterus does, and the eggs are small and numerous. Also, the cephalic spines apparently are not as closely associated with the oral sucker as in *D. lydiae* (Looss, 1901; Nicoll, 1909), being arranged more like those of the echinostome collar and lacking the two rows of smaller spines in the ventral gap.

Dihemistephanus brachyderus Manter, 1940, is in agreement with the family diagnosis in most respects although it has two ascending excretory tubules on each side formed by the division of each main tubule a short distance from the vesicle. We have examined a specimen of *D. brachyderus*, kindly provided by Professor Manter, and found that the female complex is typical of the Acanthocolpidae; a Laurer's canal is present and the beginning portion of the uterus is expanded to form a prominent receptaculum uterinum, a true seminal receptacle being absent. In all other respects except the ascending excretory tubules, which may represent an aberrant condition, *D. brachyderus* is typically acanthocolpid in structure, although its allocation to the genus *Dihemistephanus* is somewhat uncertain, as stated by Manter (1940). On the whole, however, *D. brachyderus* resembles *D. lydiae*, the type species, far more than does *D. sturionis*.

The descriptions of *Lepidauchen hysterospinosa* Manter, 1931, and *Pleorchis sciaenae* Yamaguti, 1938, extend our knowledge of genera hitherto imperfectly understood, so that they now may be included in the family Acanthocolpidae. Also, *Pseudolepidapedon* is unquestionably an acanthocolpid. *Lepidauchen* and *Pseudolepidapedon* differ from the

original genera of the family chiefly in respect to the terminal genitalia, a character whose taxonomic significance is often unreliable and has been greatly overestimated in proposing supergeneric groups. The presence of intestinal diverticula and multiple testes in *Pleorchis* are of not more than generic significance (compare *Fasciola* and *Fasciolopsis*; *Siphodera* and *Exorchis*). Since the genera *Pleorchis* and *Schistorchis*, for which Poche (1925) erected the family Pleorchiidae, do not seem to be at all closely related, this family must be judged invalid.

Although the acanthocolpids bear certain resemblances to the echinostomes, as noted by many authors, life history studies indicate that their closest relatives are to be sought among the allocreadiid trematodes having ophthalmoxiphidiocercariae. These include *Allocreadium*, *Crepidostomum*, *Megalogonia*, *Bunodera* and related genera. The family Allocreadiidae² should be restricted to include these forms. However, the acanthocolpids differ from the allocreadiids in morphology and host-parasite relationships. As far as known at present, allocreadiid cercariae develop in freshwater bivalves and encyst in invertebrates while acanthocolpid cercariae develop in marine gastropods and encyst in fishes. The chief morphological difference is in respect to the excretory system. In view of these differences, the Acanthocolpidae and Allocreadiidae are regarded for the present as distinct families of a common superfamily.

The systematic position of the genus *Deropristis* remains to be discussed. There seems no alternative to the conclusion that its nearest relatives are such forms as *Homalometron*, *Anallocreadium*, *Microcreadium* and *Lepocreadium*. In our opinion, the subfamilies Lepocreadiinae and Anallocreadiinae and the genus *Deropristis* constitute a family independent of the Allocreadiidae. As far as we have been able to determine, the only published proposal of such a combination is the grouping of several genera under the heading, Lepocreadiidae, in the Zoological Record for 1934. Since this list presumably is attributable to Nicoll, the sectional editor, the family is defined below under the following heading:

² The authors' (Hunninen and Cable, 1941) recent proposal to restrict the family Allocreadiidae to contain those trematodes having in common cotylomicrocercous cercariae and certain other characteristics was based on the assumption that *A. angusticolle* was actually a species of *Allocreadium* since it did not violate the original description of the genus. The description of *A. isoporum*, the type species, was unavailable at the time our paper was revised. This description fully justifies Dobrovolsky in transferring *A. angusticolle* to *Plagioporus*; although Looss performed no life history experiments with the ophthalmoxiphidiocercaria which he believed to be the larva of *A. isoporum*, it is very likely that his belief was correct. We therefore retract our proposal in favor of that of Hopkins (1941), viz., that the name Opcoelidae be used for the restricted family.

Lepocreadiidae Nicoll, 1934

Medium sized distomes, body variable in shape, rarely without spines. Mouth subterminal, prepharynx, pharynx, and esophagus present; intestinal ceca almost always long, rarely joining excretory vesicle or with separate anal openings. Excretory vesicle tubular, variable in length, frequently reaching pharyngeal level; rarely Y-shaped and then with short arms not receiving main tubules; excretory pore posterior, terminal or subterminal. Main excretory tubules never reach cephalic region but divide near acetabular level to form anterior and posterior collecting tubules. Flame cells numerous. Genital pore anterior to ventral sucker, median or displaced to left; rarely marginal or with a "genital sucker." Cirrus sac present or absent; if present almost always followed by an external seminal vesicle. Testes in posterior half of body, occasionally multiple, usually two, median and tandem, sometimes diagonal or opposite in short bodied forms. Ovary pretesticular, rarely behind level of anterior testis. True seminal receptacle and Laurer's canal present. Vitellaria usually extensive, often filling body posterior to ventral sucker, rarely confined between levels of cirrus sac and anterior testis or restricted to post-testicular region of body. Uterus preovarian in almost all genera and not extensive; eggs relatively few and large. Parasites of fishes. Cercariae of trichocercous type, stylet lacking, with conspicuous eye-spots remnants of which may persist in adults. Develop in simple rediae in gastropods, encyst in invertebrates. Includes the following subfamilies:

Lepocreadiinae Odhner, 1905

With characters of the family. Genital pore to left of median line; cirrus sac present, followed by external seminal vesicle which is sometimes embedded in a glandular mass. Vitellaria well developed, reaching posterior end of body; uterus preovarian in almost all genera. Parasites of marine fishes. Type genus *Lepocreadium* Stossich, 1903. Includes also:

Lepidapedon Stafford, 1904 (syn. *Lepodora* Odhner, 1905).

Opechona Looss, 1907 (syn. *Pharyngora* Lebour, 1908).

Aephnidiogenes Nicoll, 1915.

Pseudocreadium Layman, 1930 (syn. *Leptocreadium* Ozaki, 1936).

Diploporus Ozaki, 1928 (syn. *Bianium* Stunkard, 1930).

Multitestis Manter, 1931.

Rhagorchis Manter, 1931.

Lepotrema Ozaki, 1932.

Mysoxenus Manter, 1934.

Lepocreadioides Yamaguti, 1936.

Hypocreadium Ozaki, 1936.

Labrifer Yamaguti, 1940.

Allolepidapedon Yamaguti, 1940.

Opechonoides Yamaguti, 1940.

Homalometrinae nom. nov. pro *Anallocreadiinae* Hunter
and Bangham, 1932

With the characters of the family. Body not very elongate, with or without spines. Genital pore median, immediately in front of or rarely posterior to ventral sucker; cirrus sac lacking. Vitellaria well developed, rarely restricted to post-testicular region. Parasites of freshwater and marine fishes. Type genus *Homalometron* Stafford, 1904 (syn. *Anallocreadium* Simer, 1929). Includes also:

Microcreadium Simer, 1929.

Crassicutis Manter, 1936.

Myzotus Manter, 1940.

An unnamed genus reported by Manter (1941).

Deropristiinae subf. nov.

With characters of the family. Body elongate and spinose with enlarged spines on the middorsal and lateral regions of forebody. Genital pore median, anterior to ventral sucker; cirrus sac present. Cirrus and metraterm with prominent spines; genital sinus tubular. Ovary and anterior testis separated by a space into which the uterus may extend. Vitellaria scanty, between levels of cirrus sac and anterior testis. Parasites of migratory fishes; larval stages marine. Type and only genus *Deropristis* Odhner, 1905.

Since the subfamilies *Lepocreadiinae* and *Homalometrinae* differ chiefly in respect to the terminal genitalia, their validity may be questionable. However, differences in their cercariae and the fact that both subfamilies have become established in the literature probably justify their retention. Manter (1926) expressed the view that the genera *Anallocreadium* and *Homalometron* probably are synonymous. We can find no basis for maintaining the separate identity of these genera. Since *Homalometron* has clear priority, the name *Anallocreadium* is not available for the subfamily *Anallocreadiinae* proposed by Hunter and Bangham (1932). In restudying Stafford's collection, Miller (1941) has expressed the opinion that *Homalometron* and *Lepocreadium* may be synonymous. We have placed these genera in separate subfamilies and fail to see the reason for Miller's opinion since *Lepocreadium* has a long

excretory vesicle and cirrus sac while *Homalometron* has a short vesicle and lacks a cirrus sac. Incidental to the present study, we have determined that the excretory system of *H. pallidum* is very similar to that of *Deropristis*, the chief difference being a much larger number of flame cells in *Homalometron*, particularly in the anterior groups.

The trematodes assigned by various authors to the families Gyli-
auchenidae and Opistholebetidae present a perplexing problem which
Manter (1940) has discussed recently. Travassos (1934) and Ozaki
(1937) have assigned these families to the superfamily Paramphisto-
moidea, although they contain genera ranging from the usual distome
type to forms superficially resembling the amphistomes. Figuring largely
in this disposition was the presence of lymphatic vessels in certain genera.
Manter (1940) pointed out the resemblance of certain gyli-
auchenids to the Lepocreadiinae and Anallocreadiinae and described what appeared
to be lymph channels in species of *Opechona* and *Pseulepidapedon*. On
the other hand, he cites Carassotrema, a gyli-
auchenid, which lacks lymphatic vessels, indicating that their presence does not necessarily denote
amphistome affinities. Manter therefore concluded that the family Gyli-
auchenidae probably should be considered in the superfamily Allo-
creadioidea.

A study of Ozaki's (1937) excellent descriptions of the gyli-
auchenids affords convincing evidence that they are aberrant forms closely related
to the family Lepocreadiidae. Their peculiar body shape can be ex-
plained by failure of the hindbody to grow during post-cercarial develop-
ment, with a compensating elongation of the forebody and a resultant
shifting of certain internal organs anteriorly. The so-called excretory
papilla then would correspond to the post-acetabular region of ordinary
distomes. Several morphological features support this explanation.
First, the genera *Petalocotyle*, *Telotrema*, *Flagellotrema* and *Gyli-
auchen* comprise a fairly complete series of body forms ranging from the usual
distome type to one resembling the amphistomes. In this series, there is
a progressive elongation of the prepharynx and decrease in the length
of the intestinal ceca until they are little longer than the pharynx in
*Gyli-
auchen*. There is also a progressive decrease in the length of the
excretory vesicle and retreat of the testes from the hindbody which be-
comes too small to contain them. Elongation of the forebody is sug-
gested not only by the length of the prepharynx, but also by the distance
separating flame cell groups near the middle of the body; the genital pore
and ventral sucker become widely separated. Furthermore, the lym-
phatic vessels which extend posteriorly only to the ventral sucker or about
one-fourth the body length in *Petalocotyle*, likewise reach the ventral
sucker in *Telotrema* but are consequently almost as long as the body in

this species. Progressive reduction of the hindbody is indicated not only by the shortening of the intestinal ceca and excretory vesicle, but also by the manner in which the flame cell groups are crowded in the hindbody of the intermediate form, *Telotrema*, and apparently become reduced in number in *Flagellotrema*.

The genital complex and excretory system of the gyliachenids agree well with those of the lepocreadiids and certainly are unlike those of typical amphistomes. In no case, is the ovary posterior to the testes as in most amphistomes and only in *Flagellotrema*, where it lies between the testes, does it approach the condition found in certain short-bodied amphistomes. The reduction and anterior displacement of the vitellaria may be correlated with the shortening of the intestinal ceca and compensating elongation of the prepharynx. The somewhat shortened ceca and arrangement of the reproductive system in the lepocreadiid *Opechonoides* are suggestive of certain gyliachenids.

On the basis of structure, it could be argued that either the amphistome or the distome is the primitive gyliachenid body type. This question cannot be answered until the larval stages are discovered. In view of the fundamental morphological resemblances of the gyliachenids to the lepocreadiids, we believe that the family Gyliachenidae does not belong in the Paramphistomoidea but instead in a common superfamily with the Lepocreadiidae. Further study may in fact necessitate the combination of these families if it is shown, as some observations indicate, that the lymphatic system in trematodes is correlated with physiological requirements that may arise independently in various groups.

Secondary acquisition of amphistome characteristics in the family Opistholebetidae is suggested by the series *Maculifer*, *Heterolebes*, and *Opistholebes*. There is some suggestion that this group may be related to the Opecoelidae but the evidence is not sufficiently clear to postulate affinities. This is true also of the family Cephaloporidae.

It is uncertain whether the families Allocreadiidae (*sensu stricto*), Opecoelidae, Lepocreadiidae, Acanthocolpidae, and Gyliachenidae represent only one or more than one superfamily. Our knowledge of life histories and embryological development, particularly of marine forms, is still fragmentary and it is possible that in certain groups the few life histories known are of species having atypical larval stages such as have been described in other groups and would by themselves be misleading as a basis for judging relationships.

SUMMARY

The life history of *Deropristis inflata* has been demonstrated experimentally. The cercaria is a trichocercous form developing in simple

rediae in the marine snail, *Bittium alternatum*. It encysts in *Nereis virens* and the eel, *Anguilla rostrata* serves as the natural definitive host which becomes infected by eating annelids containing metacercariae.

The genus *Deropristis* and *Dihemistephanus sturionis* are excluded from the family Acanthocolpidae which is redefined to include for the first time the genera *Lepidauchen*, *Pleorchis*, and *Pseudolepidapedon*. The family Pleorchiidae thereby becomes invalid.

It is proposed that the family Allocreadiidae be restricted to include only forms known or believed likely to have ophthalmoxiphidiocercariae with main excretory tubules not reaching the cephalic region of the body before receiving secondary tubules. The Allocreadiidae and Acanthocolpidae are regarded as separate but closely related families.

The genus *Anallocreadium* is reduced to synonymy with *Homalometron* and the subfamily Anallocreadiinae is renamed Homalometrinae. The Lepocreadiinae, Homalometrinae, and new subfamily Deropristiinae are regarded as a distinct family, the Lepocreadiidae. The family Gyli-*au*chenidae is believed to be nearer the Lepocreadiidae than the Paramphistomoidea. Whether the Allocreadiidae, Acanthocolpidae, Opecoelidae, Lepocreadiidae and Gyli-*au*chenidae constitute only one or more than one superfamily is not apparent from the fragmentary knowledge of life histories and embryology.

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STUDIES OF THE RESPIRATORY METABOLISM OF WARM AND COOL SPRING FISHES¹

F. B. SUMNER AND URLESS N. LANHAM

I. INTRODUCTION

It is generally agreed that the respiratory metabolism (oxygen consumption) of poikilothermal animals tends to vary directly with the temperature to which they are subjected. Less familiar is the existence of a process of thermal adaptation, following transfer from one temperature to another. The immediate effects of such transfer may be succeeded by a "rebound" from the extreme condition first induced back toward the previous condition of the animal. A further illustration of the same principle is the fact that the metabolic rate of fishes at any given temperature may be considerably influenced by their previous temperature experience. Fishes which had been acclimatized to 30°, for example, showed a lower rate of oxygen consumption, when transferred to 20°, than ones which had been kept continuously at 20°, and conversely, transfer from 10° to 20° resulted in a higher rate than that shown by fishes accustomed to the latter temperature. Here, too, any such departure from the normal oxygen consumption for a given temperature is followed, within a few days, by a process of adaptation which tends to equalize the metabolic rate, regardless of previous history (Wells, 1935*b*; Sumner and Wells, 1935; Sumner and Doudoroff, 1938).

An obvious question is whether the metabolic rate of fishes and other poikilothermal animals in nature varies as widely with the temperature of their medium as might be inferred from these laboratory results. Or, may not more far-reaching processes of adaptive modification occur in nature, such that warm-water and cold-water derivatives of the same stock come to have nearly the same rates of metabolism?

Fox and Wingfield² compared certain closely related species, or local representatives of the same species, living at different latitudes, and reported varying degrees of thermal adaptation of this sort. In some cases, there was no such adaptation, the two forms under comparison differing widely under their own normal conditions of life, but approximating one

¹ Contributions from the Scripps Institution of Oceanography of the University of California, La Jolla, California, New Series No. 157. Services were rendered in the course of these studies by persons working under W.P.A. Project 65-1-07-2317.

² Fox, 1936, 1939*a*, 1939*b*; Fox and Wingfield, 1937; Wingfield, 1939.

another closely when their rates of physiological activity were compared at equal temperatures. In other cases, the rates of these functions were nearly identical in their respective habitats, despite considerable differences of temperature. Here extensive adaptation appears to have taken place.

In 1939 Sumner and Sargent made field studies of the respiratory metabolism of fishes in springs of varying temperature in Nevada. Experiments were conducted upon warm and cool spring representatives of species belonging to two genera, *Cyprinodon* and *Crenichthys* (Sumner and Sargent, 1940). In these studies, the rapidity of death in thousandth-normal KCN was used as an index of the rate of respiratory metabolism, the two, as is well known, being directly correlated.

In both of these genera, the warm-spring fishes, at their accustomed temperature, succumbed far more rapidly than the cool-spring fishes at their accustomed temperature. As regards the behavior of warm-spring individuals, transferred to the cool spring, the two fishes seemed to give different results, though the data hardly warrant definite conclusions on this point. It should be pointed out, however, that the seeming lack of adaptive modification of the metabolic rate on the part of *Crenichthys* in warm water has been borne out by the studies to be reported herewith. Its striking resistance to the lethal effects of high temperature is something quite distinct (Sumner and Doudoroff, 1938).

The importance of the physiological and ecological problems here involved, and the widespread human threat to the continued availability of warm springs to naturalists (Brues, 1928) seemed to warrant a second trip to the springs previously visited in our studies of *Crenichthys*. This work was carried on during portions of September and October, 1941.

While we believe that the publication of our present results calls for no apologies, it should be pointed out that field studies of this sort should not be judged by the same standards as are applied to similar studies in the laboratory. This is because of the impossibility of transporting important articles of laboratory equipment, unfavorable weather conditions, and limited time (in this case, less than 4 weeks) at the scene of our operations.

We must here acknowledge valuable help received at various times from our colleagues, Dr. Denis L. Fox and Dr. Marston C. Sargent, and from our companion in camp, Mrs. Margaret Sumner.

II. METHODS

Altogether, 200 fishes were employed in these studies, while more than 200 oxygen determinations were made. The fishes were readily caught

at both of these springs with hand-nets. Some time (several hours to a day or more) before the respiration tests were made, small lots (6 to 9) of the fishes, of roughly uniform size, were placed in each of four glass tubes having a length of 40 cm. and an internal diameter of 4 cm. Except during the period of a test, wide-mesh cloth screen was fastened over the flanged ends of the tubes, which were laid, side by side, in a screen cage, placed at the bottom of the stream, and directly in the line of the current. When a test was to be made the screens were removed from one of the tubes, and close-fitting rubber stoppers, one of these carrying a glass stopcock, were forced into its ends. The tube was then returned to its former position in the cage. The capacity of these tubes, with the stoppers in place, ranged from 439 to 454 ml.

At the close of 15 minutes (10 in some experiments) the water-samples were drawn off into glass-stoppered bottles of about 150 ml. capacity. A glass tube, continuous with the stopcock, was inserted into the bottle, down to its bottom, and the water allowed to overflow from the bottle's mouth. Two samples were invariably drawn in each test.³ Time records, in seconds, were kept of each of these steps with a stop-watch. Within a few minutes of the drawing of each sample, the Winkler reagents were introduced into the bottles. Titration was performed on either the same or the next day.⁴

In recent studies of oxygen consumption in fishes (cf. Keys, 1931; Wells, 1935a; Wunder, 1936; Leiner, 1938) it has been the more usual practice to hold the animals in a continuous current of water, and to draw samples from this at intervals, rather than to hold them, tightly stoppered, in an inclosed volume of water. Under the former conditions, the accumulation of carbon dioxide and other possible metabolites is, of course, prevented. Likewise the fishes are subjected to less disturbance in the taking of water samples. It is obvious, however, that such procedure would be difficult, if not impossible, to employ in the field. Moreover, the highest of our CO_2 concentrations probably fell well short of two thousandths of an atmosphere. No signs of discomfort were visible at the close of the 15-minute period, at least in the cooler water. So far as disturbance is concerned, the fishes showed little evidence of excitement except at the beginning and the end of the test. Most of the time they swam quietly or remained at rest. In this connection, it is worth pointing

³ The values for oxygen consumption employed in our tables and graphs have been based entirely upon the first of these samples. The second figures have been regarded as less representative for a number of reasons.

⁴ No appreciable effect of delaying the titration over night is probable. Readings of four divided samples, titrated on the same and on the following day, gave mean values of 2.48 and 2.52 respectively. These were as close as the average divided samples titrated on the same day.

out that our figures show no more variability for fishes of a given size than do those of Wells (1935a, pp. 209–210) in experiments with *Fundulus*, where a running-water system was employed. Indeed Wells has recorded fully as great differences as ours when repeating tests of *the same lots* of fishes.

Before and after the performance of a series of these tests, and frequently alternating with them, water samples were withdrawn from the spring, as near as possible to where the tubes had lain.

Owing to the possible presence of nitrites in our samples (cf. Allee and Oesting, 1934) the modified Winkler method (A.P.H.A., 1936)⁵ was used in all cases, with a few exceptions which were performed for the sake of comparison.⁶

Under field conditions, it is hardly to be expected that such high precision will be attained as is possible in the laboratory. Thirty cases in which duplicate water samples were drawn from a single tube revealed a mean difference of 2.2 per cent between them. Lack of homogeneity ("streakiness"), in the water, to be discussed presently, may have had its effect, even within our half-liter tubes.

At the close of each test of oxygen consumption, the fishes involved were dried upon paper towels and weighed. The aggregate weight of each lot, together with the estimated original oxygen titre of the water, the oxygen titre at the close of the test, the volume of water in the tube (allowing for volume occupied by the fishes), and the exact duration of the test, are the data upon which the metabolic rates (ml./gm./hr.) are based.

Unfortunately, the equipment at our disposal did not permit of our collecting a sample of the water admitted to the tube at the moment of commencing each test of oxygen consumption. For this reason, the previous oxygen titre of the water which was actually "breathed" by the fishes in any given experiment cannot be stated with precision. As will appear later, water samples from these springs, taken at different times or in slightly different positions, might differ considerably in their oxygen titre. While this circumstance introduces a considerable margin of possible error into the result of any single titration, we believe that the validity of our chief comparisons is little affected. This (1) because the

⁵ Our procedure differed from the procedure therein stated in that in the succeeding steps of the test (Winkler proper) HCl was used instead of H₂SO₄, while manganous chloride was used in place of the sulphate. This is in accordance with the procedure long employed at the Scripps Institution in analysis of sea-water.

⁶ It is doubtful whether the modified procedure was necessary, in the case of the water-samples taken directly from the springs. Four divided samples, of which half of each was subjected to the modified and half to the unmodified Winkler procedure, gave mean values of 2.54 and 2.55 respectively.

differences involved in comparing two series of differing history are great in proportion to errors due to uncertain original oxygen content of the water; and (2) because results based upon two different procedures which we have employed in selecting a figure to represent this oxygen content show substantially the same relations.

The most obvious of these procedures (1) was to select for each computation the oxygen value of that water sample which was taken most nearly at the same time as the performance of the metabolism test under consideration. However, there were instances in which no water sample was taken within an hour or more of this test.

The alternative procedure (2) was to employ, as our basis of calculation, the mean titre of all the water samples taken in the position in which the test in question was made.⁷ It is obvious that many single values for oxygen consumption obtained in this way would be no more than rough approximations. It is equally plain, however, that the *average* of a series of values thus obtained could not depart far from an average such as we should obtain if the actual oxygen titres of the corresponding water samples were available.

It is gratifying that graphs based upon these two procedures agree in most essential features, though differing considerably in detail (Figs. 2-4). In the text, the mean of the two resulting values will be given.

A further difficulty in postulating any initial oxygen content for the water is the fact that a quite appreciable amount of oxygen was taken out of their medium by the fishes before the commencement of the test, despite the continuous flow of water through the tubes. This current was undoubtedly retarded somewhere by the screens, which it was necessary to fasten over the ends. Whatever the exact extent of this preliminary depletion of the oxygen, we may probably fairly assume that it did not differ very widely from one experiment to another.

III. THE SPRINGS

"Preston Spring," the largest of several springs in the vicinity of Preston, Nevada, is really a group of small springs, discharging near together and giving rise to a creek of considerable size. The water in and near the orifices appears to maintain a constant temperature very close to 21° C.

The high variability of the oxygen values for all of the water samples taken at Preston Spring is indicated by Fig. 1. Reference has already been made to the "streakiness" of this water in respect to oxygen content.

⁷ Excluding those taken on cloudy days or near sunset, which regularly gave very low values.

While the concentration at the sources appears to be fairly constant (cf. Sumner and Sargent, 1940), it is considerably lower than that in the rest of the stream, even in their immediate neighborhood. Surface aeration and photosynthesis are, of course, responsible for the differences. The mean of two samples, drawn from what appeared to be the largest orifice was 1.96 ml. per litre, while samples taken a few yards away averaged about 2.60 ml. The resulting variegation in the oxygen concentration of these waters was shown by determinations of samples taken in various positions.

It would be quite erroneous, however, to infer that the water with which we filled our tubes for the tests of respiratory metabolism was

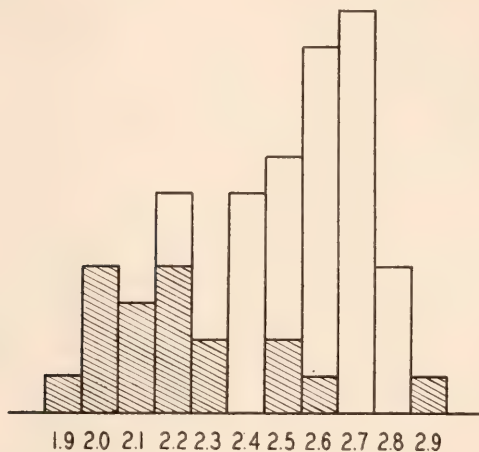


FIG. 1. Oxygen values (ml./l.) for water of Preston Spring. Shaded squares denote samples taken in the first position of our cage directly over one of the minor orifices of the spring; the others in a second position some distance from this.

subject to such an extreme range of variation as is here indicated. Care was taken, moreover, to collect our samples from points close to those where the tests were made.

Some thirty miles to the south, in the same valley,⁸ is the warm spring here discussed, known locally as "Mormon Spring." This is a spring of much smaller volume than that at Preston. Its temperature is almost exactly 37° at its source, but the water cools off by a degree or two within a hundred feet downstream. Most of the fishes were caught in water ranging from 35° to 36.5° C., and temperatures such as these prevailed where the experiments were performed. At the lowest point in the stream where fishes were seen, the thermometer recorded 34.8°. Within

⁸ Called the "White River Valley," though there is no continuous stream here.

a few hundred feet from its source, the stream spreads out into a marsh, where the water probably follows the temperature of the air. No fishes were found there. It seems probable, therefore, that the Mormon Spring representatives of *Crenichthys baileyi* seldom experience temperatures lower than 34°. On the other hand, individuals were rather frequently seen close to the main source, where the temperature was nearly or quite 37°.

As regards oxygen content, water samples taken well below the surface, in the main orifice of the spring, gave a mean value of 0.51 ml./l., while at a nearby point, where fishes were often seen, this value was 0.65. Samples taken downstream in the creek, where most of the fishes were caught, ranged from 1.54 to 1.88.⁹

IV. THE FISHES

Crenichthys baileyi (Gilbert)¹⁰ is the commonest species of fish in Preston Spring, at least near the sources. Besides *Crenichthys*, there are, however, at least three other fishes present. Two of these (*Apocope osculus* and *Lepidomeda sp.*) are Cyprinidae, the other (*Pantosteus sp.*) being a Catostomid. At Mormon Spring, *Crenichthys* is the only fish present. Likewise, it is much less abundant than in Preston Spring. Dr. Hubbs informs us that he regards the two populations of *Crenichthys* here considered as subspecifically distinct. He states (cf. Hubbs and Kuhne, 1937, referring to similar differences in *Apocope*) that the warm spring form has a slightly lower average number of fin-rays than the cool spring form, and counts which we have made of the dorsal and anal rays of about fifty fishes of each race confirm this statement.

Identification of the sexes is difficult or impossible in the smaller specimens mostly used in these studies. Since such individuals were probably sexually immature, it is not likely that sexual differences occurred in their metabolic rate.

V. OXYGEN CONSUMPTION

Experiments were performed in which each stock was tested in its own waters, and in which each was tested after transfer to the habitat of the other.

Preston at Preston

When all of these fishes are thrown together as a single population (Fig. 2 and Table I), the values for respiratory metabolism (ml./gm.-

⁹ Readings for these were as follows: 1.54, 1.56, 1.62, 1.76, 1.78, 1.86, 1.86, 1.88.

¹⁰ Cf. Hubbs, 1932; Hubbs and Miller, 1941. We are indebted to Dr. Hubbs for the identification of the other species.

/hr.) are seen to vary very widely, ranging from 0.123 to 0.390, using in each case the mean of the values obtained by our two procedures (see p. 317). This variation is in part due to the heterogeneous nature of the material. The series includes fishes differing widely in size, this ranging from 0.51 gm. (mean of one lot of 9 individuals) to 9.5 gms. (weight of one large individual tested alone). While the (inverse) correlation between size and metabolic rate is not very close, Figs. 2 and 3 show that

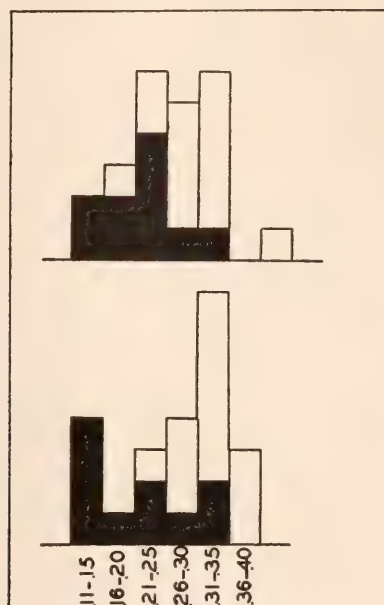


FIG. 2. Rate of oxygen consumption (ml./gm./hr.) of Preston Spring fishes in Preston Spring. Upper histogram is based upon computations (1) in which the O_2 value of the water sample taken at most nearly the same time as the test was used in each case; lower histogram upon computations (2) in which the mean of the entire series of O_2 values (with certain exceptions) was used (see p. 317). Shaded squares denote rate for fishes larger than 1 gram in mean weight, others for fishes less than 1 gram.

such correlation exists. It will be found, too, that the mean of ten values for fishes averaging over 1 gm. in weight is 0.215, that of the thirteen values for fishes below this mean weight being 0.307 (see Fig. 2).

In two cases, special experiments were made with Preston-at-Preston lots, in order that more strict comparisons with the Mormon-at-Mormon series might be possible. Thus, in the case of four lots (designated by asterisks) the period of the test was abbreviated to ten minutes (see be-

low) instead of fifteen minutes as was the rule in the Preston Spring fishes. Again, four other lots (designated by daggers) were tested only a few hours ($1\frac{1}{2}$ to 4) after being confined in the tubes, instead of being previously kept through one night, as was commonly done. While the results of both of these tests indicated considerable differences from the general average (13 to 18 per cent), part of this difference appears to

TABLE I

Oxygen Consumption of Fishes (First Sample Drawn)—Preston Spring
(Arranged in order of mean weight)

Lot of fishes	Weight of sample	Number of fishes	Mean weight	ml./gm./hr.		
				Procedure 1	Procedure 2	Means
A	4.6	9	0.51	0.370	0.424	0.328
B	5.1	9	0.57	0.341	0.307	
C	5.1	9	0.57	0.278	0.200	
D†	3.5	6	0.58	0.358	0.348	
E†	3.8	6	0.63	0.357	0.271	0.314
F	6.6	9	0.73	0.253	0.293	0.273
G	5.1	6	0.85	0.283	0.212	0.264
G'*	5.1	6	0.85	0.268	0.293	
H†	5.4	6	0.90	0.309	0.303	0.314
I	5.4	6	0.90	0.306	0.236	
I'*	5.4	6	0.90	0.333	0.343	
J	5.5	6	0.92	0.326	0.317	
J'*	5.5	6	0.92	0.334	0.329	0.239
K	7.0	6	1.16	0.153	0.222	
L†	7.0	6	1.17	0.343	0.239	0.252
M	7.8	6	1.30	0.277	0.270	
M'*	7.8	6	1.30	0.311	0.308	0.229
N	8.0	6	1.33	0.144	0.204	
O	8.7	6	1.45	0.236	0.222	0.218
P	10.0	6	1.67	0.209	0.227	0.192
Q	11.2	6	1.87	0.179	0.205	0.130
R	5.4	1	5.40	0.150	0.111	0.144
S	9.5	1	9.50	0.136	0.153	
Means			1.56	0.272	0.262	0.267

have been due to the size of the fishes employed. In any case the chief relations here considered are little affected.

(2) *Mormon at Mormon*

It was difficult for practical reasons (distance and time) to leave the Mormon Spring fishes over night in tubes before making the tests of oxygen consumption in their own habitat, as was customary in the

Preston-at-Preston series. Likewise it was found that the usual fifteen minutes after stoppering was too long a period of confinement, considering the high temperature ($36^{\circ} \pm$) of the water and its low oxygen content.

Two preliminary lots, however, were subjected to this longer test. The values for oxygen consumption for these lots (mean weights 0.76 and 0.67 gm.) were 0.386 and 0.382, ml./gm./hr., respectively. Some of these fishes showed marked signs of asphyxiation during the last few minutes. That respiratory values for partially asphyxiated fishes cannot be regarded as normal is obvious.

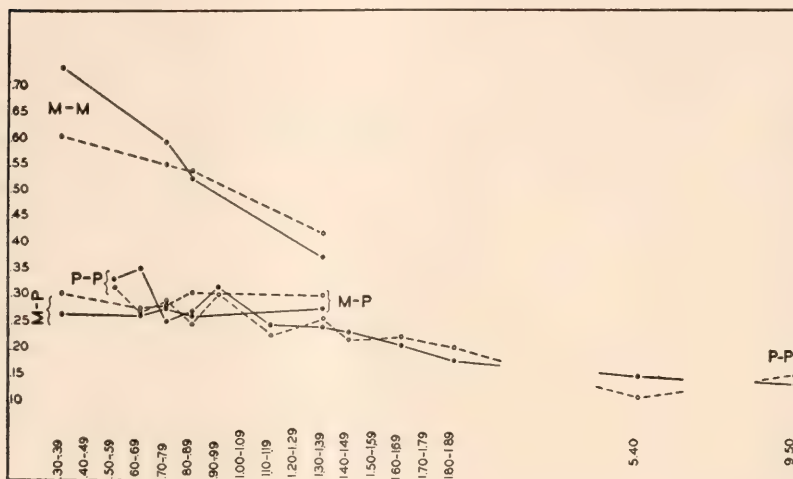


FIG. 3. Comparison of "Preston-at-Preston" series with "Mormon-at-Mormon" and with "Mormon-at-Preston" (1 day). Abscissas = mean weight of fishes in each lot; ordinates = oxygen consumption (ml./gm./hr.). Solid lines connect values obtained by second of above procedures (see legend for Fig. 2); broken lines connect values obtained by the first procedure.

The remaining four tests, limited to ten minutes, are very instructive. In Table II (upper section) it is seen (1) that values for oxygen consumption are far higher in this series than in the "Preston at Preston" series; (2) that these values are inversely correlated with the mean weights of the fishes involved. This correlation is far more evident than in the Preston series, a significant fact which will be discussed shortly. Comparisons between the Mormon-at-Mormon and Preston-at-Preston series may be made by inspection of Fig. 3. That the relations shown here are little affected when we substitute certain more strictly comparable Preston-at-Preston lots, has already been pointed out.

(3) *Preston at Mormon*

It had already been reported by Sumner and Sargent (1940) that Preston Spring fishes speedily die when transferred to Mormon Spring. In the more recent experiments, it was found that the fishes were overcome so rapidly by the heat that no instructive determinations of oxygen consumption were possible.

(4) *Mormon at Preston*

In these experiments, the fishes were transferred from Mormon Spring to Preston Spring in partially insulated bottles. The temperature fell seven or eight degrees in the course of transfer, though this circum-

TABLE II

	Lot of fishes	Weight of sample	Number of fishes	Mean weight	ml./gm./hr.		
					Procedure 1	Procedure 2	Means
Mormon at Mormon	T	3.5	9	0.39	0.739	0.612	0.675
	U	4.4	6	0.73	0.597	0.555	0.571
	V	5.1	6	0.85	0.528	0.544	0.536
	W	8.3	6	1.38	0.382	0.423	0.402
	Means			0.84	0.561	0.533	0.546
Mormon at Preston (2½ to 4 hours)	T'	Same values			0.422		
	U'				0.374		
	V'				0.328	0.342	0.335
	W'				0.255	0.263	0.259
	Means				0.345		
(1 day)	T''	Same values			0.268	0.308	0.288
	U''				0.256	0.288	0.272
	V''				0.266	0.308	0.287
	W''				0.278	0.304	0.291
	Means				0.267	0.302	0.284

stance would seem to have little bearing on the results. Three different series of these fishes (8 lots, comprising 57 fishes) were thus transferred. The most instructive of these series consisted of the same four lots as were employed in the principal Mormon-at-Mormon tests. The further history of these fishes is included in Table II, and is portrayed graphically, in part, in Fig. 4. After the test in Mormon Spring, these fishes were left over night in the latter, in the same tubes (screened at the ends). They were then taken in bottles to Preston Spring and tested,

first, after the lapse of $2\frac{1}{2}$ to 4 hours following transfer; next after a day. As regards their performance after this briefer period, we have complete figures only for the second of our procedures in computing oxygen consumption. The mean of these four values is 0.345, which is plainly intermediate between 0.561 (before the transfer) and 0.267 (one day following the transfer).

The significant features to be observed in the behavior of these four lots of fishes at the close of a day following transfer are (1) the great fall in metabolic rate in the cooler water; (2) the inverse correlation be-

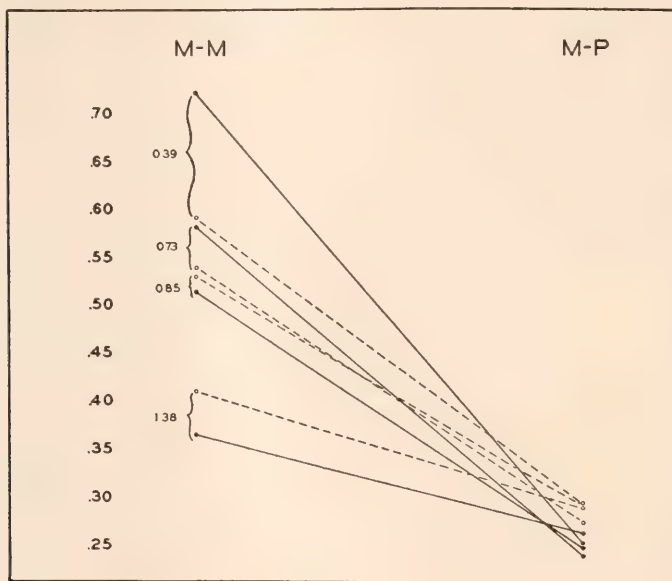


FIG. 4. Rate of oxygen consumption of same group of Mormon Spring fishes: (1) at Mormon Spring; (2) after 1 day at Preston Spring. Ordinates = ml./gm./hr. Solid and broken lines have same significance as in two preceding figures. Figures opposite brackets denote mean weights of the fishes of these respective lots.

tween the size of the fishes and the rate of this fall, resulting in a marked convergence of the curves for the four size-groups, which come to nearly a common level by the end of 24 hours.

A question of considerable interest is whether the present figures give evidence that the respiratory metabolism of these warm-adapted fishes, when transferred to much cooler water, undergoes any such fall below the rate shown by cool-adapted specimens as had been shown in previous laboratory experiments.

At the close of the day our mean figure for the metabolic rate of six lots of Mormon-at-Preston fishes was 0.284; that for the Preston fishes in their natural habitat being 0.267. The mean weight of the Mormon-at-Preston fishes (0.79 gm.) is, however, much less than that of the Preston-at-Preston fishes (1.56 gms.), a fact which might, perhaps, account for this higher rate. Graphs for these two groups (Fig. 3), in which each is plotted according to weight, make it plain that any possible difference of metabolic rate, in either direction, is small in extent.

It cannot, therefore, be affirmed with any probability that these warm-spring fishes, when transferred to water at 21°, display a rate of oxygen consumption which is lower, at the close of one day, than fishes native to the latter. This conclusion may be regarded as confirming the results of the earlier test with the cyanide method, already referred to.

Two of the eight Mormon-at-Preston lots comprised in our various experiments were kept in the latter spring for two more days.¹¹ The figures for these two lots (mean weight 0.52 and 0.57 grams) were 0.283 and 0.343. While not very consistent, these figures certainly do not indicate any further fall of metabolic rate.

The convergence of the four pairs of curves in figure 4 is a striking feature of this graph and deserves some comment. At the close of a day in the cool spring, the differences between these four lots, in respect to metabolic rate, have virtually disappeared, or have even been slightly reversed. Thus, the correlation between size and metabolic rate, which is so conspicuous at 36°, has vanished at 21°. It has already been pointed out that this correlation is low in the cool-spring fishes themselves.

A marked divergence of the temperature-O₂-consumption curves for "large" and "small" *Fundulus* is shown by Wells (1935a, Figs. 3 and 4). Wells's observations relate to temperatures ranging from 10° to 24°, so that the case is not altogether parallel with our findings on *Crenichthys*, in which we are dealing with water at 21° and 36°, and with no intermediate values. In the case of our warm-spring fishes, too, the correlation actually disappears at the lower temperature. It is plain, however, that the two cases are in essential agreement in one important respect.

That the metabolic rate of organisms declines with age, or conversely, that a higher metabolic rate is to be expected in younger animals, appears to be an accepted fact (Child, 1915; Heilbrunn, 1937). Since fishes are known to grow more or less continuously through life, it is usually a safe assumption to regard relative size as an index of relative age. Our

¹¹ Some specimens were kept for four and even six days, but these were weighed in the meantime. Since a number of these fishes died as a probable result of the unavoidable drying, the results cannot be regarded as significant.

series of fishes of increasing weight (Table I) may therefore be safely regarded as representing, on the whole, a series of increasing age.

Concerning the correlation between age and the temperature-coefficient, less appears to be known. The majority of workers seem to have reported that this coefficient increased with age (Bělehrádek, 1935). Our figures, on the contrary, as well as those of Wells, certainly indicate a higher temperature coefficient for the *smaller* fishes.

SUMMARY

Fishes (*Crenichthys baileyi*) living in a warm spring at temperatures of 35° to 37° have a far higher rate of oxygen consumption (ml./gm./hr.) than fishes of the same species living in a cool spring (21°). The ratio, when fishes of approximately the same weight are compared, is not far from 2 to 1.

Cool spring fishes, when transferred to the warm spring, die so rapidly that measurements of oxygen consumption are not practicable.

When the converse experiment is performed (transfer of warm-spring fishes to the cool spring), they live in health for some days, and may perhaps do so indefinitely. Changes in their rate of oxygen consumption are as follows: (1) A rapid fall occurs within the first few hours. (2) At the close of a day, the metabolic rate (oxygen consumption) is about the same as that of fishes native to the cool spring. If any difference exists, when specimens of the same size are compared, this is probably slight.

This failure of the metabolic rate of warm-spring fishes to fall below that of cool-spring fishes, when transferred to the latter medium, accords with previous experiments at these same springs in which the cyanide method was used, but it does not accord with experiments performed upon some other species in the laboratory.

A marked inverse correlation exists between size (age) and metabolic rate. This correlation is more marked at high temperatures than at lower ones. Thus the curves for the four size-groups of warm-spring fishes here considered converge strongly during the test in cool water, coming together at nearly a common point by the end of the day. This last fact harmonizes with the fact that the correlation between size and metabolic rate, while observable, was found to be low among the cool spring fishes.

The oxygen consumption of smaller fishes is much more affected by temperature changes than is that of the larger specimens. In other words, the temperature coefficient is higher for the smaller (younger) animals, a fact which does not appear to agree with most results which have been reported for other organisms.

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THE MECHANISM FOR SYNCHRONOUS SPAWNING IN HYDRACTINIA AND PENNARIA

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INTRODUCTION

Synchronous spawning of all individuals in one neighborhood at a particular time of day occurs in various Hydrozoa. It has been noted in *Pennaria* and *Clava* by Beckwith (1909), in *Halyclistis* and *Sertularia* by the Teissiers (1927), and in *Hydractinia* by various workers. These are all colonial species, and the sexual individuals are sessile. Moreover, only one sex is normally represented in each colony, and male and female colonies may live at some distance from each other. If all the eggs and sperm are discharged at the same moment in a given mud flat or tide pool, the chance of survival of the genus is greatly enhanced. How this happens is the subject of the present study.

There have been conflicting reports as to the regular spawning time of *Hydractinia*. Bunting (1894) observed it to be between 9.30 and 10.30 P.M., Teissier and Teissier (1927) between 7 and 8 A.M. C. W. Hargitt (1911) found spawning to occur at various times of the day but most frequently in the early morning. My own observations showed that *Hydractinia* in a state of nature always spawns shortly after sunrise. All but the Teissiers worked at Woods Hole.

Reports from many observers are unanimous that *Pennaria* sheds its eggs and sperm normally several hours after sunset (G. T. Hargitt, 1900; Baker, 1936).

Attempts to force both these species to spawn at any desired hour of day or night have now proved successful, and have opened an approach to the problem of the nature of the spawning mechanism. Reasons can be offered for the conflicting reports on *Hydractinia* and for the difference in preferred spawning time between *Hydractinia* and *Pennaria*.

METHODS OF CONTROLLING SPAWNING

Hydractinia echinata Fleming is in good breeding condition at Woods Hole in June and July. The colonies, growing on shells inhabited by

hermit crabs, may be kept in open dishes of sea water and will shed eggs or sperm copiously once a day for a week before becoming exhausted.

It was first noted that *Hydractinia*'s sunrise spawning could be delayed indefinitely by leaving the colonies in the dark of a desk drawer or loose box. When they were returned to the light they spawned in about an hour, with some variation in time according to the condition of the colonies.

Colonies continuously illuminated during the night from an ordinary reading lamp omitted the normal spawning at sunrise, and would not spawn at any later time until they had first been darkened for a while and then restored to light.

In practice, spawning was produced in *Hydractinia* at will by (1) leaving the colonies in running sea water under a glowing 100-watt light bulb from the time of collection until needed; (2) then placing them in the dark for from one to several hours; (3) then exposing them to daylight or a desk lamp, at which time the colonies could be examined with a hand lens and segregated according to sex. Practically all male colonies in prime condition would spawn 50 minutes after the moment of re-illumination (Fig. 1), female colonies five minutes later (Fig. 2).

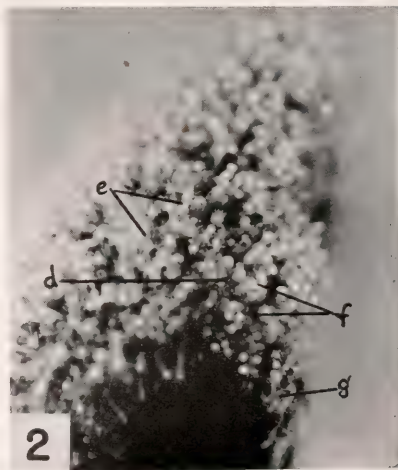


PLATE I

FIG. 1. Male colony of *Hydractinia* just starting to shed sperm. (a) Gonozooid bearing two white ripe gonophores and two translucent unripe gonophores; (b) feeding polyp; (c) spiral streams of sperm from ruptured gonophores. Toward lower right, a foggy area full of dispersing sperm. About 6 $\times$.

FIG. 2. Female colony of *Hydractinia* just starting to shed eggs. (d) Gonophores containing ripe unshed eggs; (e) feeding polyps; (f) clusters of eggs just shed; (g) stinging polyps around lip of snail shell. About 6 $\times$.

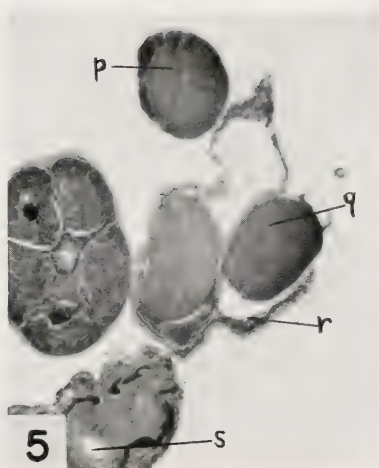


PLATE II

FIG. 3. Section of *Hydractinia* gonozooid 45 minutes before spawning. (*h*) Gonophore containing unripe eggs; (*i*) gastrovascular cavity of gonozooid; (*j*) distal end of gonozooid with nematocysts; (*k*) gastrodermal spadix of ripening gonophore; (*l*) peripherally placed enlarged germinal vesicle of ripening egg. About 60 $\times$.

FIG. 4. Section of *Hydractinia* female gonophore just before shedding. (*m*) Mature egg cell; (*n*) egg follicle; (*o*) immature oöcyte in a neighboring gonophore. About 100 $\times$.

FIG. 5. Section of *Hydractinia* female gonophore preserved during the act of spawning. (*p*) Discharged egg; (*q*) egg emerging; (*r*) collapsing wall of gonophore, showing outer epidermis and inner musculo-epithelium; (*s*) gastrovascular cavity of parent gonozooid. About 100 $\times$.

FIG. 6. Section showing three *Hydractinia* eggs in late cleavage stages still within the gonophore. Maturation induced by dark and light treatment, shedding inhibited by calcium-free sea water. About 100 $\times$.

In *Pennaria tiarella* Ayres, both uninterrupted illumination and uninterrupted darkness were likewise found to inhibit the normal evening spawning, as had earlier been suggested by Baker (1936). But after a prolonged period in the dark, spawning took place 10 to 14 hours after return to the light, irrespective of the time of day. The mechanism seems similar, but *Pennaria* reacts far more slowly to light than *Hydractinia*.

Since laboratory aquaria may be in quite dark corners, and since the turning on of lights in the laboratory may precipitate the shedding reactions, it is not surprising that earlier investigators should have reported synchronous spawning of *Hydractinia* at various times of the day. The suggestion of Bunting (1894) that heat and cold control the spawning of *Hydractinia* has not been confirmed, and her experiments in this direction are invalidated by the fact that storing the colonies in a (dark) icebox and later removing them to room temperature (and light) will produce the same result whether the icebox be warm or cold.

MATURATION PHENOMENA

Early accounts of maturation in *Hydractinia* eggs were incomplete or fallacious because material was not collected at the right time of day. Beckwith (1914) was the first to report the cytological details, from study of a quantity of material preserved at dawn. The present investigation corroborates her findings.

Whether spawning be the normal sunrise event or whether it be produced at another time by control of the lighting, the hour following re-illumination and prior to shedding is occupied in maturation. Since the preceding spawning period, the germinal vesicle has enlarged, become sharply granular and moved from a central position to the distal cell wall (Fig. 3), and it now becomes smaller and hazy, disappearing about half an hour after the light stimulus. The first polar body is given off at the same distal point in 45 minutes, the second polar body ten minutes later. The first polar body often divides. At this time, 55 minutes from the time of re-illumination in most material, the wall of the gonophore ruptures (Fig. 4) and the eggs come oozing out (Fig. 5).

Each new egg is covered by a highly transparent but radially striated jelly, which has not been mentioned by previous workers. The jelly swells considerably on contact with sea water, pulling the polar bodies away from the egg surface and pushing the egg away from the collapsing gonophore. Very soon the polar bodies are so far from the egg that they are brushed off by the least disturbance, and lost.

Dissection experiments show that the process of maturation is initiated solely by the action of light on the oöcyte itself, and not by any

general action on the colony or the individual. Single gonozooids isolated from either male or female colonies lived well for five days in watch glasses of sea water, and responded either to the succession of night and day by normal shedding at sunrise, or to controlled illumination by shedding when experimentally planned. Single gonophores isolated from the gonozooids did likewise, although it was not possible to separate in advance those which would shed from those that would not. In practice, a batch was made up by collecting the ripest-looking single gonophore from each of a number of very well developed gonozooids; depending on the actual ripeness of the parent colony, anywhere from 0 to 100 per cent of the gonophores so selected would shed their eggs after dark-light treatment.

When large numbers of apparently ripe primary oöcytes were dissected out of gonophores selected in this fashion, and these isolated oöcytes were then subjected to the routine of one hour of darkness and one hour of light, some of them could be watched passing through maturation stages according to the expected timetable, and could later undergo fertilization and cleavage. Sperm added before or during maturation were without effect until emergence of the second polar bodies. The proportion of maturing to non-maturing eggs in a large group of these dissected eggs was the same as the proportion in the control group of intact gonophores from the same colony. In other words, light could stimulate maturation in the isolated oöcyte with the same facility that it did in the normal animal.

PREPARATION BY DARKNESS

The amount of time required in the dark to prepare the *Hydractinia* colonies for shedding is certainly short, although no spawning will take place in continuously illuminated colonies. Eggs can be ripe enough so that only 15 minutes or less in the dark will prepare them for reaction to light, but a longer period is necessary for maximum yield of eggs. In a group of 36 female colonies that were allowed just 15 minutes in the dark, only one shed any eggs after re-illumination. Eighteen of these same colonies were given several more hours in the dark, and all shed heavily following the second re-illumination.

Performance of colonies that were darkened for half an hour was somewhat better. Forty-two female colonies were kept illuminated for 36 hours or more, then darkened for half an hour. Only 12 shed eggs after re-illumination. Darkness for an hour or longer followed by re-illumination usually produces spawning in 100 per cent of prime fresh colonies.

What takes place within the eggs during the dark period is a question. No nuclear changes are discernible in fresh or sectioned material. Until some time after re-illumination, it is not possible to distinguish the eggs that are going to be shed from the large unripe eggs in neighboring gonophores. Perhaps darkness allows the accumulation of light-sensitive substances whose breakdown initiates the maturation processes.

SPAWNING AFTER RE-ILLUMINATION

In a few cases, colonies kept in a box which admitted a crack of light on one side were found to have spawned before return to full daylight, but when colonies were put in complete photographic darkness after prolonged illumination, eggs were never shed in the dark. Ten female colonies were kept in complete darkness for $28\frac{1}{2}$ hours, without shedding a single egg. On removal to light, all ten colonies shed within the hour.

To test the effectiveness of a short dosage of light, 76 female colonies which had been continuously illuminated for 24 hours since they had been collected were divided into two apparently equivalent batches and stored in complete darkness for $4\frac{1}{4}$ hours. One was to shed after a brief exposure to light, the other after prolonged exposure to light, and the relative effectiveness of the exposures was to be tested by capacity for re-spawning after a second immediately succeeding period of darkness.

Batch A was illuminated for 80 seconds at 1.15 P.M. and restored to the dark. At 3.15 P.M. it was taken from the dark and the heavy spawn of eggs was removed. Left on the desk in daylight, this batch was then expected to shed more eggs in one hour, but only an insignificant several hundred eggs were produced by 5 P.M.

Batch B was left on the desk in full north window daylight from 1.15 to 2.30 P.M. Heavy shedding of eggs took place from 2.10 to 2.15 P.M., and these eggs were removed. From 2.30 until 3.45 P.M. batch B was stored in complete darkness and then placed in the window by the side of batch A. Only a few dozen eggs were produced in the second spawning before 5 P.M.

Batches A and B were kept illuminated overnight and the experiment was repeated the next day. This time batch A was given only 12 seconds of light at 1.15 P.M. Batch B stayed in the light an hour and a quarter. Both batches shed heavily, in approximately equal quantity. As before, the total elapsed time from first darkness to last illumination was the same in the two batches. After the second dark period, batch A shed only several hundred eggs, batch B shed none at all.

Eggs from each different batch were funneled into a standing column in a burette to measure their volume, and a rough estimate of their number was calculated from counts of samples of known volume. Each batch produced well over 6,000 eggs on each day. Batch B was slightly more productive than batch A in the sum of its two sheddings the first day, but there was no significant difference the second day.

It is clear that from 12 to 80 seconds of strong daylight is sufficient stimulus to bring about a heavy shedding of dark-prepared eggs, and that illumination in excess of this is able to bring about the discharge of only a scattering few more eggs. It is not at all essential that the colonies remain in the light during the period from their re-illumination until the time they are shed.

However, a very brief period of illumination depends for its effectiveness on an ample period of previous darkness. On July 18, 50 female colonies which had been continuously illuminated for over 24 hours were stored in the dark for one hour, exposed then to ten seconds of light from a 100-watt bulb two feet away, and then kept in the dark for $2\frac{3}{4}$ hours. No eggs whatever were shed, but after standing for an hour longer under the light bulb, a heavy spawn resulted. On the following day, 34 of these same colonies, after 13 hours of illumination, were given an hour in the dark, ten seconds of daylight (north window, midday), and $1\frac{1}{2}$ hours in the dark. On removal to daylight again, a moderate shedding of eggs was found, but within the hour twice as many more eggs were shed as a result of the new illumination. No explanation can be offered why a procedure closely similar to that which failed to cause shedding the first day should succeed the following day. But in both cases it is clear that ten seconds of light, which would have been sufficient to precipitate a near-maximum shedding if the colonies had been kept a long time in the dark, was far less effective after only one hour's darkness.

ROLE OF THE JELLY LAYER IN EGG SHEDDING

Since the eggs are obviously under considerable pressure inside the gonophore during their maturation phase, and since their jelly layer expands while they ooze outward, it was considered whether the jelly was not the immediate agent in rupturing the gonophore and inducing shedding, simply by the absorption of water. Several considerations make this appear unlikely.

Immature eggs are coated with a dense but entirely transparent layer of jelly tightly confined inside a very thin sphere of flattened follicle cells (Fig. 4). When oöcytes with large germinal vesicles are dissected free from the gonophore, the rupture of the follicle-cell layer causes the jelly

to swell rapidly and it begins to show very delicate radial striations. If the follicle-cell sphere remains unbroken, the jelly remains unswollen for an indefinite period. This indicates that if the swelling of the jelly causes shedding of the egg there must exist also some egg-induced rupture or altered permeability in the follicle-cell layer, if not the gonophore wall also, allowing the jelly to imbibe water.

But there is evidence that eggs can be shed from the gonophore after destruction of the jelly. Of several treatments which resulted in the dispersal of jelly, the one least harmful to eggs or polyps was immersion in calcium-free sea water. *Hydractinia* can survive without permanent ill effects in Van't Hoff's artificial sea water with CaCl_2 omitted for upwards of 14 hours, *Pennaria* for a considerably shorter period.

Hydractinia gonophores containing eggs undergoing maturation, and gonophores containing germinal-vesicle stages, were rinsed in this solution, and the eggs dissected out in it. Some eggs showed very thin and watery jelly, but most showed none at all. *Pennaria* eggs similarly dissected from the medusae in this solution were invariably obtained without jelly, whereas those dissected out in normal sea water had a thick jelly coat best seen in ink suspension. This effect having been observed, efforts were made in *Hydractinia* to disperse the jelly before shedding could start.

Ten female colonies were kept in the dark nine hours and brought to the light, being transferred at once to calcium-free sea water in which they remained one hour. Returned then to normal sea water, they recovered enough to produce a heavy spawn of eggs in another hour and a half, but practically none of these eggs showed any jelly at all. They could be packed together in contact with each other, which cannot be done with ordinary eggs unless the jelly is removed by violent shaking. These jelly-less eggs were mature, and when insensated they cleaved on schedule.

CALCIUM ESSENTIAL FOR SPAWNING

If male or female colonies of *Hydractinia* are exposed to the action of calcium-free sea water for three quarters of an hour or longer just prior to the expected shedding time, no spawning occurs in the solution, and a recovery period is necessary in normal sea water before eggs or sperm are released. The corresponding reactions in the more sensitive *Pennaria* are striking.

Pennaria has well-developed medusa-gonophores, in contrast to the degenerate sporosac-gonophores of *Hydractinia*. When *Pennaria* is

ready to spawn, the pendant medusae open their bells, and the inner musculo-epithelium of each starts a rhythmic pulsation which is so prolonged and powerful as to accomplish the discharge of the sexual products. When a colony containing many pulsating medusae is transferred to calcium-free sea water, the musculo-epithelium of each medusa is almost instantly paralyzed. Not even the slightest muscular tremor is found after one minute in the solution. Transfer back to normal sea water is followed by prompt recovery, and at the end of a minute or two the medusae are pulsating as strongly as before.

In *Hydractinia*, the inner musculo-epithelium shares in the degenerate development of the whole sporosac (Goette, 1907, Plates V, VI), and is difficult to demonstrate except during the actual process of extrusion of the eggs (Fig. 5). This and the fact that it is not directly exposed to the action of the fluid until the moment of spawning in the sporosac may explain why *Hydractinia* does not show the extreme rapidity of response to lack of calcium exhibited by *Pennaria*.

Since the calcium-free sea water has been shown to have an effect on the jelly layer around the egg, the possibility of inhibition of shedding also through direct injury to the egg must be considered.

Calcium-free sea water apparently does not hinder the darkness-preparation of the oöcytes inside the gonophores. One colony, remaining in this solution $1\frac{1}{2}$ hours in the dark, was transferred to normal sea water in the light, and produced eggs in 65 minutes. Another colony, after one hour in the dark in this solution, was illuminated then for one minute during transfer to normal sea water and darkness, and presently shed eggs in the dark.

The question whether calcium is necessary for the carrying out of the maturation processes and the initiation of them by light has not been put to critical test. However, as proof that the dosage of calcium-free sea water can be adjusted so as to allow successful maturation and yet inhibit shedding, a case of experimentally produced internal fertilization in *Hydractinia* may be cited.

Twelve female colonies were put into calcium-free sea water in the dark for one hour.* Next they spent 30 minutes in the light in normal sea water, then an hour in the light in calcium-free sea water again. No eggs having been shed at the end of that time, the gonophores were carefully scrutinized under the microscope with strong light, and the absence of germinal vesicles noted in a number of the eggs. The colonies were left in normal sea water from then on, and a few eggs were extruded in the next two hours. Examination still showing unshed matured eggs in some of the gonophores, sperm were added to the

dishes. Figure 6 shows a section through a gonophore which contained three unshed eggs in late cleavage stages, preserved three and a half hours later.

In many hydrozoan genera, such internal development of embryos is normal. Nothing like it ever occurs normally in *Hydractinia*.

DISCUSSION

Influence of light on breeding seasons or spawning reactions is well known in a number of phyla. In most animals the germ cells are well shielded from light and the action is indirect, through the nervous system or in more subtle ways. Even in the translucent ascidians whose spawning is controllable by light (Rose, 1939), some fertilizable eggs can be obtained by dissection at any time of day or night. I cannot cite any other case in which light directly initiates the maturation processes of animals.

One not altogether consistent case among the Chlorophyceae deserves mention. Kater (1929) found that *Chlamydomonas nasuta* underwent cell division regularly and almost exclusively between 9 and 12 P.M. On the other hand Moewus (1933) reports that *Chlamydomonas eugametos* undergoes cell division both in light and dark, but more cell division occurs in regular 12-hour alternations of light and dark than in light. If the cells, lighted for 12 hours, are then kept dark more than 48 hours, all cell divisions cease, even with all other conditions favoring.

Moewus presents some rather remarkable observations on light-controlled sexual activity in *Chlamydomonas*. The gametes never copulate in the dark. When brought into the light, they must be exposed for 30 to 45 minutes before they are ready to copulate. This delay, perhaps representing some sort of physiological maturation, could be reduced by soaking the unlighted gametes in water which had stood for some time in the light with other gametes of the same sexual strain in it, and then been filtered free of them. According to Moewus the soluble effective substances produced by the gametes in the light are highly sex-specific, and those of opposed sex neutralize each other. Other genera of green algae studied by Moewus revealed none of these peculiar reactions to light.

In *Hydractinia* the fact that shedding of eggs occurs only in a few of the large numbers of gonophores in a colony, and the fact that only mature eggs are shed, suggests that the actual shedding process is initiated by some late-maturation change in the eggs themselves. Whether this is a mere increase in volume which ruptures the gonophores, or a chemical stimulation of the musculo-epithelium, or what, is the subject of further experiments now in progress.

SUMMARY

1. Methods are reported for scheduling the shedding of eggs and sperm by *Hydractinia* and *Pennaria* at any desired time of day.

2. Maturation in these animals occurs only after the germ cells have undergone a period of preparation in the darkness, and is initiated by direct action of light on the germ cells. Once initiated by light, maturation can proceed in the dark. Uninterrupted light, or uninterrupted darkness, results in complete cessation of maturation and spawning.

3. In *Hydractinia* maximum shedding requires at least an hour's darkness-preparation, although less time is sufficient for some eggs. Ten seconds of illumination is sufficient for widespread but not maximum initiation of maturation.

4. The hypothesis is advanced that darkness allows the accumulation of light-sensitive substances within the germ cell, whose breakdown initiates the process of maturation. Oöcytes dissected free from the organism can be matured by appropriate dark-light treatment.

5. Shedding of eggs and sperm takes place simultaneously with the completion of the maturation process. The swelling of jelly around the egg is not an important factor in bringing about shedding. Evidence is presented that contraction of the gonophore wall is an important factor.

6. Paralysis of the gonophore's musculo-epithelium by calcium-free sea water inhibits the shedding of mature eggs and sperm. Fertilization and cleavage of eggs within the gonophore have been produced in *Hydractinia* by causing maturation, inhibiting shedding and adding sperm.

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A QUANTITATIVE STUDY OF THE INTERRELATIONSHIP OF OXYGEN AND HYDROGEN ION CONCENTRATION IN INFLUENCING TUBULARIA REGENERATION

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Increased hydrogen ion concentration of sea water has an inhibitory effect on *Tubularia* regeneration (Komori, 1933; Miller, 1939; Goldin, 1942). Goldin presented evidence supporting the view that the effectiveness of oxygen in regeneration is limited by the inhibitory action of accumulated acid metabolites. In addition, experiments in which the hydrogen ion concentration of the sea water was increased, while the oxygen tension was maintained at a constant level, showed that there is a pH range in which regeneration is retarded, and a critical pH below which regeneration will not occur. It was also indicated that the critical pH is related to the oxygen tension.

Thus the ability to form primordia appears to be dependent upon the hydrogen ion concentration as well as the oxygen tension (Barth, 1938a, 1940) of the sea water. These experiments were designed in order to study the interrelationship of these factors in their effects on *Tubularia* regeneration. The author wishes to thank Professors L. G. Barth and H. B. Steinbach for their guidance during the course of these investigations.

METHODS

The general procedure for selecting the *Tubularia* stems, treating them and measuring rate of regeneration, was essentially the same as that described by Barth (1938a, 1938b). Young, unbranched stems of uniform diameter were selected from young colonies. Stem segments 6 mm. in length were used for the experiments, the required length being removed from the region 5 to 11 mm. proximal to the hydranth. In order to insure uniformity of material, random samples of these stem segments were taken for each experiment.

The oxygen tension and the hydrogen ion concentration of the sea water were adjusted by saturating the sea water with oxygen-carbon

dioxide-nitrogen gas mixtures. The gases were drawn from cylinders and the mixtures were prepared in a graduated 20 gallon carboy by the standard method of water displacement. The carboy was filled with water, and the water was then displaced by the required quantity of each gas. Adjustment to atmospheric pressure was made after the addition of each gas, so that the total pressure was one atmosphere. The nitrogen gas was used in order to bring all of the mixtures to the same total volume. For each experiment a series of gas mixtures was prepared containing carbon dioxide at increasing partial pressures and oxygen at the same partial pressure. Measured samples of filtered sea water (350 ml.) were introduced into one liter florence flasks and then saturated with the required gas mixtures. In this way, for each experiment, the hydrogen ion concentration (carbon dioxide tension) of the sea water was varied, while the oxygen tension was maintained at a constant level. The various experiments, however, were conducted at different levels of oxygen tension, so that a wide range of oxygen and hydrogen ion concentration was covered. Twenty stem segments were introduced into each flask, and samples of sea water were removed in order to determine the oxygen tension and the pH. Dissolved oxygen was determined by the Winkler method, and the hydrogen ion concentration was measured with a Beckman pH meter. The experimental flasks were kept at constant temperature ($19 \pm 1^\circ$ C.) during the above operations and throughout the course of the experiments.

The stems were then examined for regeneration. They were removed from the flasks as the primordia were formed, by means of a long pipette. The time was noted, and the primordia were measured with the aid of an ocular micrometer mounted in a binocular microscope. The formula $R = L/t = \text{R. U.}$ (regenerative units) was used for measuring rate of regeneration, where L is the length of the primordium in micra, and t is the time in hours at which the primordium becomes constricted from the stem segment proper. At low oxygen and high hydrogen ion concentration many of the stems did not regenerate. Stems which failed to regenerate were removed from the flasks at the close of the experiment (106–120 hours), and placed in running sea water. The stems which regenerated after removal were considered to be inhibited, and the length of their primordia as well as their rate of regeneration were considered as zero. The stems which failed to recover were not included in the calculations. This treatment of the data is in general the same as that employed by Barth (1938a), and appears to give the most accurate index of regeneration rate.

RESULTS

The results are summarized in Table I, and the rate of regeneration is plotted against pH at the different levels of oxygen concentration in

TABLE I
*The interrelationship of oxygen and hydrogen ion concentration
in influencing Tubularia regeneration*

Initial pH	Change in pH	Initial O ₂ cc./l.	Change in O ₂ cc./l.	<i>L</i> micra	<i>t</i> hours	<i>L/t</i>
<i>Experiment 1</i>						
8.00	-0.17	8.4	-1.0	1246	31.3	39.8
7.00	+0.03	8.8	-1.3	969	43.8	22.1
6.60	+0.11	8.5	-1.1	629	65.5	9.6
6.64	+0.08	7.9	-1.1	630	66.4	9.5
6.30	+0.13	8.2	-0.9	0	106.0	0
<i>Experiment 2</i>						
8.03	-0.12	11.1	-2.2	1693	28.8	58.8
7.52	-0.10	10.3	-2.0	1491	34.4	43.3
7.33	-0.01	9.8	-1.4	1623	33.6	48.3
6.97	+0.15	10.5	-1.9	1571	37.5	41.9
6.74	+0.25	10.2	-1.7	1406	40.4	34.8
6.26	+0.04	9.7	-1.2	1085	68.0	16.0
<i>Experiment 3</i>						
8.00	-0.08	10.3	-2.2	1346	30.6	44.0
6.96	+0.15	10.5	-2.4	1230	37.8	32.5
6.69	+0.17	9.9	-1.7	995	56.3	17.7
8.00	-0.08	5.6	-0.1	921	41.6	22.1
6.96	+0.13	5.3	+0.2	643	69.1	9.3
6.69	+0.16	5.1	+0.3	255	70.5	3.6
<i>Experiment 4</i>						
8.07	-0.03	6.5	-0.7	1054	33.2	31.7
7.07	+0.28	6.3	-0.3	848	46.4	18.3
6.82	+0.28	6.0	-0.5	699	71.2	9.8
6.63	+0.25	6.0	-0.7	29	91.0	0.3
8.10	-0.04	3.4	+1.3	786	52.3	15.0
7.44	+0.14	3.5	+1.1	577	58.3	9.1
7.16	+0.26	3.5	+1.3	426	78.8	5.4
7.00	+0.21	3.6	+0.6	53	81.0	0.7
6.81	+0.17	3.4	+0.6	0	120.0	0
6.64	+0.15	4.0	+0.8	0	120.0	0

Figure 1. In Figure 1 and in the text the data are presented in terms of initial pH and initial oxygen concentration. The hydrogen ion concentrations remained fairly constant during the course of the experiments, the greatest pH change being 0.28 units. The greatest change

in oxygen occurred at the high oxygen concentrations. However, as shown by Barth (1938a), at high oxygen the rate of regeneration changes slowly as the oxygen is varied, so that this probably does not enter as an important factor. The pH range studied was from 8.10 (normal sea water) to pH 6.26. The levels of oxygen concentration used extended from approximately 3.5 to 10.5 cc. O_2 per liter (normal sea water contains 4.5-4.8 cc. O_2 /l.).

As the pH of the sea water is lowered, the rate of regeneration falls off at all levels of oxygen concentration. At the highest level of oxygen concentration studied (Exp. 2; $O_2 = 10.5$ cc./l. approx.) there was a

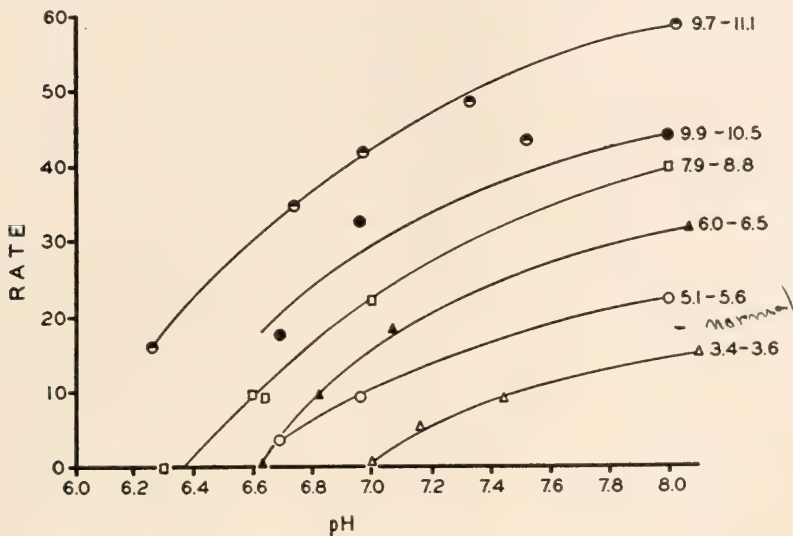


FIG. 1.¹ The interrelationship of oxygen and hydrogen ion concentration in influencing *Tubularia* regeneration. Data presented in Table I. Exp. 1 = □; Exp. 2 = ●; Exp. 3 = ● and ○; Exp. 4 = ▲ and △. Rate = L/t where L = length of primordium in micra and t = time in hours. The pH values represent the readings at the beginning of the experiments. The figures to the right of each curve indicate the range of initial concentrations in cc./l.

fall of 42.8 R. U. as the pH was lowered from 8.03 to 6.26. At the lowest level of oxygen concentration (Exp. 4; $O_2 = 3.5$ cc./l. approx.), the fall was from 15 R. U. (pH 8.10) to 0 (pH 6.81). As the level of oxygen concentration is increased, however, the rate of regeneration is increased, not only at the pH of normal sea water but at all of the pH values studied. It should be noted that stems collected at different times, even though selected for uniformity, vary in their susceptibility

¹ The author wishes to thank Mr. Jack Godrich for his assistance in the preparation of the figure.

to oxygen changes (Barth, 1938a), so that the results of the individual experiments are only approximately comparable. The trend with increased oxygen is nevertheless quite clear. In the last two experiments the regeneration of stems from the same source was studied at two levels of oxygen tension so that the results are strictly comparable. Thus, in Experiment 3, increasing the oxygen concentration from 5 to 10 cc. O_2 /l. increased the rate of regeneration 21.9 R. U. at pH 8.00, 23.2 R. U. at pH 6.96, and 14.1 R. U. at pH 6.69.

Complete inhibition of primordia formation may likewise be determined by both the oxygen and hydrogen ion concentrations of the sea water. Barth (1938a) found that the lower limit for regeneration in sea water at normal hydrogen ion concentration (pH 8–8.2 approx.) is between 0.35 and 1 cc. of oxygen per liter. As the hydrogen ion concentration is increased, complete inhibition occurs at higher oxygen concentrations (Table I; Figure 1). At pH 7.00 almost complete inhibition occurred at 3.6 cc. O_2 per liter, while at pH 6.30 complete inhibition occurred at 8.2 cc. O_2 per liter.

DISCUSSION

An increase in the oxygen concentration of sea water results in an increased rate of regeneration. This observation was made by Barth (1938a), and is confirmed by the experiments reported here. However, as the hydrogen ion concentration is increased, there is a resultant decrease in the rate of regeneration. That the oxygen and hydrogen ion concentrations are interrelated in their effects is shown clearly by the results (Table I; Figure 1). The ability to form primordia is limited by the relative concentrations of both these factors in the sea water.

The inhibitory effect of low pH is not dependent upon the source of the hydrogen ion. In the experiments reported here the change in hydrogen ion concentration was brought about by increasing the carbon dioxide tension of the sea water. Inhibition is also obtained when the pH of the sea water is regulated by means of acid, base, and buffer (Goldin, 1942).

The interaction of these two environmental factors may likewise determine whether regeneration will occur or be completely inhibited. For, as the hydrogen ion concentration of the sea water is increased the oxygen concentration required for regeneration is also increased. Thus the complete inhibition of regeneration which has been obtained after various treatments is clarified. Morgan (1903) found that the cut end of a *Tubularia* stem fails to regenerate when it is placed in sand. Ligat-

ing the cut ends of stems inhibits regeneration. Barth (1938a) and Rose and Rose (1941) demonstrated that the covering of the cut end of a *Tubularia* stem with a glass capillary is sufficient to cause inhibition. Inhibition is also obtained when coenosarc fragments of *Tubularia*, as well as stem segments, are inserted in glass tubing (Goldin, 1942). The inhibition in all of these cases may be attributed to both lack of oxygen and increase of hydrogen ion concentration. The inhibition of stem regeneration in glass tubing occurred at pH 6.9, while stems kept in open dishes (4.4 cc. $O_2/l.$) failed to regenerate in the neighborhood of pH 6 (Goldin, 1942). It was suggested that this difference could be accounted for if, in the glass tubing, a decrease in the oxygen concentration of the sea water occurred concomitantly with the accumulation of acid metabolites. This interpretation is supported by the experiments reported here, for almost complete inhibition occurred at pH 7.0 when the oxygen concentration was reduced to 3.6 cc. $O_2/l.$

The results lend support to the conclusions of Goldin (1942) on the origin of polarity in *Tubularia* regeneration. The conditions of oxygen and hydrogen ion concentration which are required for regeneration may likewise account for the origin of polarity. The polarity of dedifferentiated coenosarc fragments (Goldin and Barth, 1941; Goldin, 1942) as well as stem segments (Komori, 1933; Miller, 1937, 1939) may be determined by subjecting them to a differential environmental exposure. The differential exposure may consist of a gradient of oxygen availability, a gradient of hydrogen ion concentration (or CO_2), or a combination of both of these factors. The effectiveness of the gradients would be dependent upon the resultant concentrations of both oxygen and hydrogen ion at the exposed surfaces of the coenosarc.

In order to proceed with studies of the chemical processes involved in regeneration it is necessary to understand fully the physiological action of these environmental factors. Oxygen apparently plays an important role in the respiratory mechanism involved in regeneration. Barth (1940) studied the oxygen consumption of *Tubularia* stems, and showed that there exists a close correlation between oxygen consumption and the rate of regeneration. As the oxygen tension is increased, the tissues consume more oxygen, and the rate of regeneration is increased. Further, distal levels of the stem regenerate more rapidly and have a higher rate of oxygen consumption than proximal levels. The close relationship between the effects of oxygen and hydrogen ion concentration indicates that the same processes are affected. In this connection it would be important to test whether increasing the hydrogen ion concentration (or carbon dioxide tension) of the sea water decreases the rate of oxygen consumption.

SUMMARY

The regeneration of *Tubularia* stem segments was studied in sea water at varying concentrations of hydrogen ion (carbon dioxide tension) and oxygen. As the hydrogen ion concentration was increased there was a fall in the rate of regeneration. On the other hand, as the dissolved oxygen was increased, the rate of regeneration was increased.

The effects of hydrogen ion and oxygen are interrelated, the resultant rate of regeneration being determined by the relative concentrations of both these factors in the sea water.

Complete inhibition may be effected by increased hydrogen ion, decreased oxygen, or by a combination of both of these factors. As the hydrogen ion concentration was increased, complete inhibition occurred at higher oxygen concentrations.

The origin of polarity in *Tubularia* regeneration was discussed in terms of these results.

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THE EFFECTS OF TEMPERATURE GRADIENTS ON THE PUPAL-ADULT TRANSFORMATION OF SILKWORMS

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There is good evidence that the adult development of the higher holometabolous insects is evoked by one or more factors arising in the anterior end of the larva and pupa (Hadorn, 1941; Scharrer, 1941). In the present investigation further information was sought concerning the possible action of such a differentiation center in the pupal-adult transformation of silkworms (Williams, 1940). By the application of temperature gradients, one end of the pupa could be exposed to a temperature ordinarily high enough to induce development and the other end to a temperature ordinarily low enough to maintain dormancy. The developmental dependency of the two ends and of the various tissues could thus be tested.

There is apparently only one previous record of the use of temperature gradients in studying insect development; namely, the investigation of Giersberg (1929) concerning the pigmentation of Lepidoptera.

MATERIALS AND METHODS

The experiments were performed on two species of native silkworms, *Samia walkeri* (*cynthia*) (F. & F.) and *Telea polyphemus* (Cram.). The diapausing pupae were collected during winter, after having been exposed to low temperature, and were stored at 3° to 5° C. until used. Since escape from diapause is facilitated by prolonged exposure to cold (Uvarov, 1931), it may be presumed that the pupae were in a physiological state to develop when heated. A further advantage to the use of such material was the fact that all individuals at the beginning of the experiments were at the uniformly low degree of adult differentiation characteristic of diapausing pupae.

The apparatus consisted of an incubator from which the glass in the hinged door had been removed and substituted by a corrugated cardboard diaphragm. The pupae, in various orientations, were fitted snugly into holes of suitable size in this partition, the incubator set at a favorable developmental temperature (25° to 30° C.), and the entire

apparatus placed in a cold-room having a temperature of 3° to 5° C. A beaker of water inside the incubator maintained the humidity at a high level.

Most of the animals were oriented with the diaphragm midway of their longitudinal axes. There was thus established a temperature gradient along the body of each individual. Fortunately, no major neutralization of this effect would be expected from the circulation of the blood due to the very rudimentary state of this system in the diapausing pupa.

The animals were exposed to the temperature gradient for a maximum of 139 days. During this time the pupae which showed signs of development were removed and studied. Most of the animals were photographed and fixed in Bouin's solution. A few were exposed to room temperature to test its effect on subsequent development.

EXPERIMENTAL RESULTS

Head and Thorax Heated—Abdomen Chilled

As can be seen from Table I, development took place in all pupae thus oriented, with the exception of two individuals that died. Striking differences, however, were found in the degree of adult differentiation at the two ends. After exposure to the temperature gradient for

TABLE I

Development attained by pupae exposed to longitudinal temperature gradients

	Front heated; abdomen chilled	Abdomen heated; front chilled
No development.....	0	6
Front moulted; abdomen immature.....	21	0
Abdomen moulted; front immature.....	0	5 ¹
Both ends immature		
Front more mature than abdomen....	4	0
Abdomen more mature than front....	0	15
Dead.....	2	2
	27	28

¹ Only the anterior $\frac{1}{4}$ to $\frac{1}{3}$ of pupae chilled.

an average of 58 days most of the animals moulted the pupal cuticle at the anterior end posteriorly to about the second abdominal segment. The rest of the abdomen remained firmly enclosed within the pupal cuticle from which it could be removed only by tearing the body wall. The anterior ends of these animals were wholly adult, except that the

wings were not expanded. All the adult structures of the head and thorax were well differentiated and pigmented, as shown in Figure 4. These pupal-adult animals could walk in normal fashion and move the wings slowly. Four individuals were removed from the diaphragm before the anterior end had completely transformed.

Dissection of all of the animals that had been oriented in this manner showed that some adult development had also occurred in the chilled abdomen. However, the degree of adult differentiation of the abdomen was in all cases less than that of the anterior region. The large pupal midgut was frequently still present, although modifications of the gut in the adult direction had clearly taken place. For example, slight convolutions had appeared in the straight hindgut of the pupa and a terminal rectal expansion had been differentiated. The genitalia had initiated development, but were still immature. The first stages in the formation of the adult abdominal cuticle were apparent, although the abdominal muscles were still attached to the pupal cuticle.

Thus it is clear that development in the adult direction had begun at both ends in animals heated anteriorly and cooled posteriorly. The anterior warm end attained full or extensive development whereas the chilled posterior end remained in an earlier stage of adult differentiation.

Head and Thorax Chilled—Abdomen Heated

Six of the 28 pupae oriented in this fashion underwent no development whatsoever, although their abdomens were exposed to the warm temperature for an average of 97 days. At the end of this time these individuals were still alive, but unchanged externally and internally (Fig. 1).

Twenty pupae initiated development. For most of these animals adult differentiation was still incomplete at both ends after an exposure to the temperature gradient for an average of 74 days. Here again the warm end showed the more extensive adult differentiation, although the contrast between the developmental conditions at the warm and cold ends was not as great as when the gradient was the reverse. The adult cuticle of the abdomen was in most cases well chitinized, while that of the head and thorax less mature. Furthermore, fully developed adult genitalia occurred on animals whose thoracic muscles were incompletely formed. It may be noted also that in every case where the earliest stages of differentiation of the adult had begun in the abdomen, adult development had also been initiated in the head and thorax.

In only five instances did the abdomen become wholly mature and moult during the term of the experiments. These animals had been

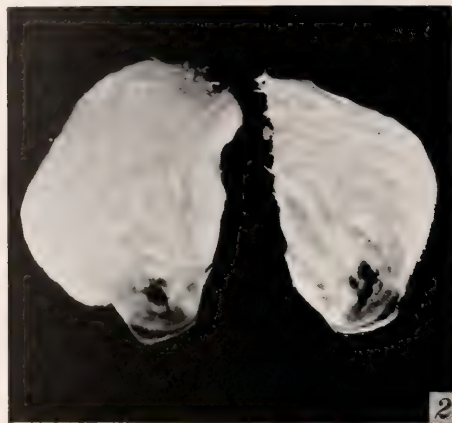


PLATE I

treated with a less severe temperature gradient, however, since only one-fourth to one-third of each individual was placed on the cold side of the diaphragm, in contrast to the usual orientation of the diaphragm midway of the longitudinal axis. The pupal cuticle of the abdomen became thin and transparent in these five animals so that it was easily removed, whereas the cuticle of the head and thorax remained firm and could be dissected off only with difficulty. A comparison of the opposite ends showed that the anterior region had attained the adult condition also, except that pigmentation and finer histological maturation were generally absent. As shown in Figure 3, all the adult structures of the head and thorax were present but generally soft and incompletely chitinized. The heated abdomen had completely transformed into the adult condition.

In summary, the results obtained from animals chilled anteriorly and heated posteriorly indicate that the development of the abdomen is retarded or prevented by cooling the head and thorax. In those cases where the abdomen became fully developed and moulted, the head and thorax were in the last stages of adult differentiation.

One Side Chilled—Other Side Heated

Pupae treated in this manner produced animals which were wholly adult, except that development proceeded somewhat more rapidly on the warm side. This was especially clear in regard to the degree of pigmentation: the hot side attained complete pigmentation before this process had scarcely begun on the chilled side. A conclusive test of this orientation was impossible, however, due to the difficulty in establishing a sufficiently steep temperature gradient transversely through the relatively narrow pupa.

PLATE I

FIG. 1. Diapausing pupa of *S. walkeri*.

FIG. 2. Hind wings of an individual (*S. walkeri*) that had been heated posteriorly and chilled anteriorly. The wing tips, which were on the warm side of the diaphragm, have become pigmented, whereas the chilled anterior region remains in an earlier developmental condition.

FIG. 3. Result of chilling the anterior region of *S. walkeri* and heating the posterior region. Both ends have transformed, but the chilled anterior region is less mature than the heated abdomen.

FIG. 4. Result of heating the anterior half of *T. polyphemus* and chilling the posterior half. The heated region has completely transformed and moulted, whereas the chilled end remains firmly attached to the pupal cuticle. The wings and legs also show gradients in development.

Subsequent Effects of Developmental Temperature

Certain of the animals that had moulted the pupal cuticle at one end were removed from the diaphragm and placed at room temperature. In about ten days a second partial moult occurred in which the rest of the pupal cuticle was shed to produce the complete adult.

At such a time those animals which had previously moulted the abdominal cuticle showed curious movements of the abdomen: waves of contraction passed forward along its length in a manner similar to that which occurs during the normal eclosion of the adult and expansion of the wings. The wings in no case were expanded, however.

Thus it was possible to produce the complete adult by two partial moults.

Local Action of Temperature

In most of the pupae the diaphragm passed across the wing flaps with the result that the tip of each wing was exposed to one temperature and the base to the other extreme. Development proceeded normally on the heated end, whereas the chilled end remained in an earlier stage of development. This was especially clear in regard to pigmentation, as shown in Figures 2 and 4. The pigmentation of the heated portion was generally complete and normal in every respect up to a narrow transitional zone of incomplete pigmentation at the former level of the diaphragm.

Other examples of local temperature action were found along the lengths of the legs and the antennae.

DISCUSSION

An analysis of the effects of temperature gradients gives us some information in regard to the initiation of adult formation after diapause. We have noted that all of the animals which began to transform showed more rapid differentiation at the warm than at the cool end; yet in no instance did development occur in the heated region without some indication of development likewise being present in the chilled region. This observation, that dormancy is apparently terminated simultaneously over the entire animal, suggests the existence of some mechanism which evokes the formation of the adult.

The experimental results permit a rough localization of such a stimulating factor. Without exception, development took place in the surviving pupae whose heads and thoraces were heated. From this we may infer that dormancy is not maintained by cooling the abdomen. In

contrast, when the anterior end was chilled, 21 per cent of the individuals underwent no development whatsoever after 97 days: overall dormancy thus persisted in these animals when the head and thorax were inhibited by the low temperature. These results may be most simply explained in terms of the existence in the anterior end of the pupa of a developmental center which must become active for adult transformation to ensue.

Further evidence in favor of the presence of such a center is given by the excessive time necessary for the development of the remaining 79 per cent of the animals chilled anteriorly. These individuals usually showed only slight differentiation after an average of 74 days. On the contrary, the head and thorax when heated became fully transformed in the majority of animals after 58 days. The fact that 21 per cent of the specimens chilled anteriorly failed to begin development after prolonged exposure suggests that the longer time necessary for adult differentiation of all animals thus treated was primarily due to a delay in the initiation of development rather than to a retardation once it had begun. If the developmental center is in the anterior end of the pupa, such a delay would be expected.

This evidence in support of the existence of an adult-differentiation center is in agreement with the results obtained by a number of previous investigators using other techniques, as recently reviewed by Bodenstein (1939). It may be noted that the action of a differentiation center in the lepidopterous pupa has been found for both diapausing (Bytinski-Salz, 1933) and non-diapausing (Hachlow, 1931; Piepho, 1938a and b; Bodenstein, 1938, 1939) types of development. The results of the present investigation indicate that the differentiation center does not exert its effect in diapausing silkworms until after a prolonged period during which development is suspended. Such an extensive delay is not found for non-diapausing species and individuals. In view of this fundamental difference it is possible that diapausing and non-diapausing developments differ primarily in terms of the time at which the center attains an activity sufficient to evoke adult development.

The local effects of temperature gradients are also of interest in regard to certain of the differentiation processes which are generally considered to be under hormonal "control." For instance, there is considerable evidence from previous studies that moulting results from the action of a hormone arising in the head of the animal (Wigglesworth, 1939; Scharrer, 1941). It is therefore noteworthy that the majority of the animals heated anteriorly moulted the pupal cuticle of the head and thorax without shedding that of the posterior abdominal region.

This local action of hormonal factors is further illustrated by those wings which showed a gradient in pigmentation. The pupal wing-flaps correspond to flattened sacs into which the blood enters from the thorax by anterior openings at their bases. Hence the chemical environment of the wing tissues, in terms of the blood, must be uniform. Now it is well known that the pigmentation of the wings involves the reaction between the scales and a chromogen circulating in the blood (Braun, 1939). Nevertheless, under the influence of temperature gradients the pigmentation generally became complete in the heated region before the process had scarcely begun in the chilled region (Figs. 2 and 4). This phenomenon was also observed by Giersberg (1929) after various regions of the wing-flaps were chilled by cold water passed through tubing.

This evidence in regard to the local action of the chemical environment shows that, at least in these cases, hormones are the servants rather than the masters of development: the ultimate result is determined by the ability of the tissues to react.

SUMMARY

1. Diapausing pupae of the silkworms, *Samia walkeri* (*cynthia*) (F. & F.) and *Telea polyphemus* (Cram.), were placed in a diaphragm and one end exposed to a temperature of 25° to 30° C. and the other end to a temperature of 3° to 5° C. There was thus established a longitudinal temperature gradient in each individual.

2. Development took place in the majority of animals thus treated. For each of these pupae evidence was found that the termination of diapause occurred simultaneously throughout the animal. This observation suggests the existence of a developmental center which evokes the overall initiation of adult differentiation.

3. An analysis of the results obtained when the head and thorax were heated with those obtained when this region was chilled permits a rough localization of such a developmental center in the anterior end of the pupa. Since a similar relationship has been noted for non-diapausing pupae by previous investigators, it is possible that diapausing and non-diapausing developments differ primarily in regard to the time at which the developmental center attains an activity sufficient to stimulate adult differentiation.

4. After the pupal-adult transformation has once been initiated, the tissues are capable of a high degree of developmental autonomy. Animals were produced which were wholly mature at the heated end while the cold end remained in an earlier stage of adult formation. This result was also observed within single organs. In such cases the develop-

mental velocity of each region was related to the local metabolic conditions as affected by the temperature gradients.

5. Of particular interest were the effects of temperature gradients upon moulting and pigmentation, processes that are generally recognized as involving the action of chemicals of a hormonal nature. The contrasting results observed in the hot and cold regions, notwithstanding their uniform blood supply, emphasizes the importance of tissue competency in carrying out hormonal reactions.

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RESPIRATION OF A COLORLESS FLAGELLATE, *ASTASIA KLEBSII*

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INTRODUCTION

There is a growing literature on protozoan respiration recently reviewed by Jahn (1941), but the sum total of research done in this field is still quite small and not comparable to the work done on respiration of bacteria, yeast and metazoan tissues. In the belief that a detailed study of the respiratory characteristics of a flagellate would have some value from the standpoint of comparative physiology, the present work was undertaken. This paper deals with the effects of various factors on the respiration of *Astasia klebsii*.

ACKNOWLEDGMENTS

I am indebted to Professor K. C. Fisher, who guided and helped me in every possible way; to Professor W. J. Kostir for suggestions and criticisms; to Professor B. S. Meyer for the use of his Warburg apparatus in a number of the experiments; and to Professor D. R. Goddard for a supply of hexosediphosphate.

MATERIALS AND METHODS

Astasia klebsii, the species used in this investigation, is a free-living, colorless, plantlike flagellate. Its nutrition is saprozoic. Food reserves of paramylum, a starch-like polysaccharide which does not respond to the usual iodine test for starch, are stored as small granules in the cell. The same strain employed in a previous investigation (Von Dach, 1940) was used.

Pure (bacteria-free) clone cultures of *Astasia klebsii* were grown at 27° C. in one liter Erlenmeyer flasks containing 500 cc. of the following organic medium:

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KNO ₃	0.5 gm.
KH ₂ PO ₄	1.5 gm.
MgSO ₄ ·7H ₂ O.....	0.1 gm.
NaCl.....	0.1 gm.
CaCl ₂	0.01 gm.
FeCl ₃	trace
Sodium acetate.....	2.0 gm.
Bacto-tryptone.....	5.0 gm.
Distilled water.....	1000 cc.

(Adjusted to pH 5.8 with N/1 NaOH or N/1 HCl)

After incubation the cells were concentrated by centrifuging, washed three times in inorganic buffer by centrifuging, and suspended in buffer. The buffers used were potassium phosphate mixtures (M/20 K₂HPO₄-KH₂PO₄) containing the following added ingredients per liter: KNO₃ 0.09 gm., MgSO₄·7H₂O 0.09 gm., NaCl 0.09 gm., CaCl₂ trace, FeCl₃·6H₂O trace. The proportions of K₂HPO₄ and KH₂PO₄ were varied according to the pH desired, after Sorenson's tables (Peters and Van Slyke, 1932, p. 816). The pH of the buffers was checked by a Hellige colorimetric comparator or a glass electrode. It was found that the cells survived longer and with less deterioration in these buffers than they did in M/20 phosphate alone.

Respiration of the cell suspensions was measured by the Warburg direct method. The standard procedures of this method, as described by Dixon (1934), were employed.

All the experiments were conducted at 25.2° C. ($\pm 0.05^\circ$). The vessels were shaken continuously at from 70 to 90 cycles per minute, through an arc of from 4.5 to 7.5 cm.; these rates were found to be wholly adequate.

A portion of each cell suspension was fixed in 10 per cent formalin for determining cell concentration. Cell counts were made by means of a Sedgwick-Rafter counting cell and a Whipple ocular micrometer. Usually between four and eight million cells per vessel were used.

At the end of an experiment the cell suspensions were removed from the respirometer vessels for the following tests: (1) pH determination; (2) microscopic examination for possible cell injury and bacterial contamination; (3) in some cases, cell counts.

In testing for cell injury and death a modification of the method of Devereux and Tanner (1927) was used. Two or three drops of cell suspension were mixed with a drop of 0.5 per cent erythrosin on a slide and examined. Cells which did not stain and which displayed movement, whether swimming or protoplasmic contraction, were regarded as uninjured. Cells which did not move at all and which nearly always

became stained were regarded as irreversibly injured or dead. Repeated tests have shown the validity of this criterion for *Astasia*.

No increase in cell number was observed to occur during an experiment. Except where otherwise noted, the number of injured astasias remained negligible, and the pH of the suspensions remained constant within 0.2 or 0.3 unit, throughout the experiments.

The number of bacteria present in the cell suspensions was negligible in all cases. While slight bacterial contamination undoubtedly occurred as soon as the pure cultures were exposed to the air, the process of quickly washing the cells and suspending them in inorganic buffer kept bacterial growth at a minimum. These experiments, then, may be regarded as essentially bacteria-free.

The gas space of the vessels contained air in all cases. Most of the experiments were conducted at pH 5.8; this pH had previously been found most favorable for growth (Von Dach, 1940). Most of the experiments were of two or three hours' duration. Results were calculated as cubic millimeters of oxygen consumed per hour per million cells, or as cubic millimeters of carbon dioxide produced per hour per million cells. In nearly all cases, the values for duplicate respirometers did not differ from each other by more than 7 per cent.

RESULTS

I. *Respiration in Inorganic Medium*

The growth of *Astasia klebsii* cultures was found to be similar to that of yeast and bacteria. In newly-inoculated cultures there occurred in succession: (1) the "logarithmic growth phase" (which usually lasted about 120 hours), during which growth proceeded at a constant maximal rate until a cell concentration of about 300,000 cells per cc. was reached; (2) the "phase of negative growth acceleration," during which growth continued briefly at a decreasing rate; (3) the "stationary phase," during which the cell population remained fairly constant at approximately 650,000 cells per cc. for at least five weeks, with no appreciable amount of cell division or cell death.

In these experiments it was not convenient to regulate the initial cell concentrations of the culture; since duration of the logarithmic phase must increase as the initial cell concentration is decreased, it would not be enough to merely state the ages of the various cultures here. Classification of cultures as to phase of growth was based on a study of their growth curves.

Figure 1 shows the relation of rate of oxygen consumption in inorganic medium to time. Experiments on several cultures in the loga-

rithmic phase and several in the stationary phase are represented. These data show the following general relationships:

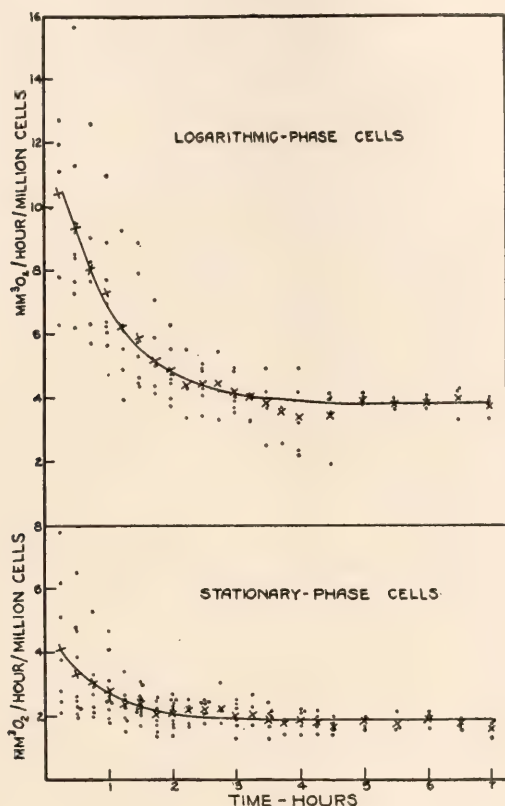


FIG. 1. Change in rate of respiration in inorganic buffer with time (pH 5.8). Dots, values from separate experiments; crosses, average values. (Logarithmic-phase cells from cultures 86 to 162 hours old; stationary-phase cells from cultures 188 to 504 hours old.)

For both old and young cells, an early period of declining respiration was followed by a more or less constant period. In logarithmic-phase cultures (i.e. "young" cells) there was an average oxygen consumption of 10 cu. mm. per hour per million cells for the first observation (at 15 minutes). The rate of respiration declined sharply for two hours, then remained fairly constant at about 3.8 cu. mm. O₂ per hour for several hours. In stationary-phase cultures (i.e. "old" cells), on the other hand, the initial average rate of oxygen consumption was 4 cu. mm. per hour per million cells; the rate declined gradually for two hours, then re-

mained fairly constant at a value of 1.9 cu. mm. O₂ per hour for several hours. Respiration rates differed somewhat even in cultures of the same age and growth phase.

Oxygen consumption in inorganic buffer was found to be approximately the same at hydrogen-ion concentrations between pH 4.5 and pH 7.9. (Results of typical experiments are shown in Table 1.)

TABLE 1
Effect of pH on respiration
(Duration of experiments three hours; M/40 sodium acetate used)

Exp. no.	Growth phase of cells	Oxygen consumption as cu. mm. O ₂ /hour/million cells		
		pH 4.5 inorganic acetate	pH 5.8 inorganic acetate	pH 7.9 inorganic acetate
1	Logarithmic	4.59	4.91	5.20
2	Stationary	2.49	2.43	2.48
3	Stationary	5.75*	2.63 31.39	
4	Stationary		2.24 28.09	29.28

* Much cell injury.

Several experiments were performed to determine the respiratory quotient in inorganic buffer at pH 5.8. Most of the determinations of rate of carbon dioxide production were carried out without the customary addition of acid (see Dixon, 1934), since it was found that there was no appreciable accumulation of retained carbon dioxide in the cell suspensions under the conditions of these experiments. The respiratory quotient was usually found to be slightly less than one. (R. Q. values obtained in four three-hour experiments: 0.86, 0.92, 0.95, 1.04.)

II. *Effects of Various Organic Compounds*

In order to throw light on the nature of the substances and reactions involved in the respiratory metabolism of *Astasia*, the effect of various organic compounds on oxygen consumption was studied. Most of the substances tested serve as substrates for many types of cells; others are known to play some accessory part in certain cellular respiration systems. (See Needham and Green, 1938, for a recent account of the role of these compounds in cellular respiration.)

Usually 0.18 cc. of a M/2 test solution was added to 1.62 cc. of cell suspension in a Warburg vessel, and the respiration compared to that of control vessels containing cells suspended in inorganic buffer alone. Cultures of various ages were employed in these experiments. In most cases cultures in the stationary phase of growth were used. In each

separate experiment, oxygen consumption in the presence of the tested compound was expressed in terms of percentage of the control respiration. Results are shown in Table 2.

A definite increase in oxygen consumption was produced by the following: sodium formate, sodium acetate, sodium propionate, and ethyl alcohol (all M/20 concentrations), and M/40 sodium hexosediphosphate. Acetate had the most marked effect, with propionate and ethyl alcohol producing somewhat smaller increases. Rather slight but consistent in-

TABLE 2

Effect of various organic compounds on respiration
(M/20 concentrations used unless stated otherwise. Nearly
all experiments of two hours' duration.)

Substances tested	Respiration as per cent of that in inorganic medium in separate experiments	Respiration as per cent of that in inorganic medium (average)
Sodium formate.....	192; 147; 151; 137	157
Sodium acetate.....	488; 731; 1130	783
Sodium propionate.....	428; 328; 229	328
Sodium butyrate.....	492;* 174;* 46;* 66*	195*
Sodium hexosediphosphate (M/40).....	172; 163	168
Ethyl alcohol.....	833; 355; 278	489
Sodium citrate.....	115; 68; 99	94
Sodium beta-glycerophosphate.....	120; 86; 96	101
Sodium lactate.....	111; 113; 106	110
Sodium succinate.....	118; 96; 103	106
Galactose.....	132; 100; 110	114
Levulose.....	102; 113; 84	100
Glycerol.....	110; 127; 105	114
Dextrose.....	98; 107; 104	103
1-Xylose.....	96; 103; 129	109
Methyl alcohol.....	99; 127	113
Ethylene glycol.....	102	102
Potassium malonate (M/500).....	100	100
Potassium fumarate (M/500).....	99	99

* Much cell injury.

creases in respiration occurred in the presence of formate and hexosediphosphate. Inconsistent results were obtained with M/20 sodium butyrate; in some experiments it produced an increase in respiration, in other cases a decrease. Butyrate always caused injury or death in from 50 per cent to 90 per cent of the total cells present; the greater degree of injury was correlated with lower oxygen consumption. Various common substrates which are oxidized by many types of cells, such as simple sugars, lactate, succinate, etc., had no appreciable effect on the respiration of *Astasia*.

Since acetate produced the greatest increase in oxygen uptake, and since it has been shown to greatly increase growth of plantlike flagellates in general (Hall, 1941) and of *Astasia* in particular (Von Dach, 1940), the effects of this substance on respiration were studied in some detail.

Respiration in Acetate

At pH 5.8, when a solution of sodium acetate was tipped from the side-arm of a Warburg vessel into the main compartment containing

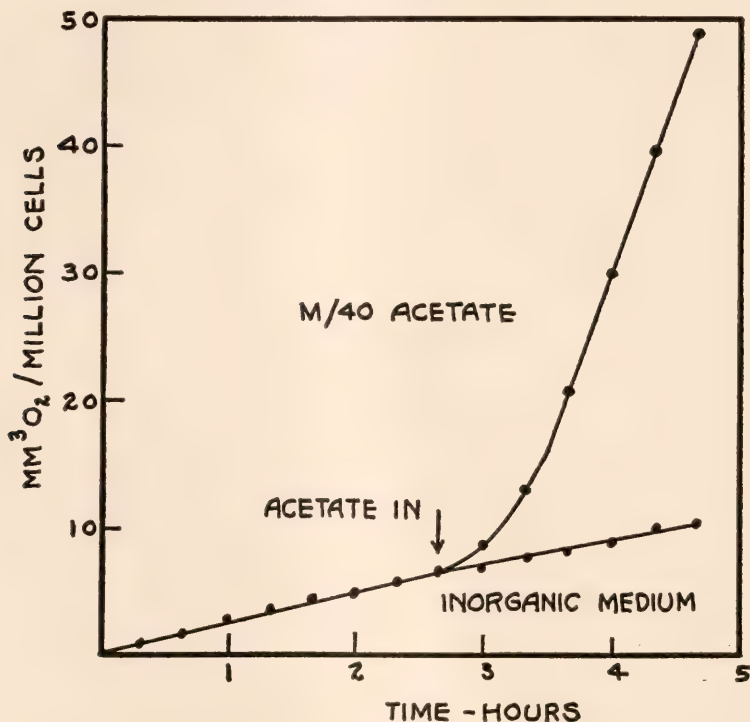


FIG. 2. Effect of adding acetate to cells in inorganic medium at pH 5.8. Results of a typical experiment. First 160 minutes: curve represents average respiration in four vessels containing cells in inorganic medium. M/40 acetate was added to two of the vessels at 160 minutes, at the arrow mark. From 160 to 280 minutes: upper curve represents average respiration in the two vessels to which acetate was added, lower curve represents average respiration in the two control vessels to which nothing was added.

cells in inorganic buffer, the rate of respiration rose steadily during a period of about one hour. (See Figure 2.) Thereafter respiration usually continued at a constant maximal rate for several hours. The acetate respiration data in this section deals with constant maximal rates.

Oxygen consumption in acetate was approximately the same at pH 5.8 and pH 7.9; but at pH 4.5 respiration was much less, and there was marked cell injury (Table 1). This differs from respiration in buffer alone, where oxygen consumption was approximately the same over the range pH 4.5–pH 7.9.

At pH 5.8 oxygen consumption was for a time approximately the same at acetate concentrations between M/20 and M/80 (see typical results in Table 3). However, after some time respiration in M/80 acetate declined rapidly, while that in M/20 and M/40 remained constant. It may be presumed that the enzyme system concerned was at first "saturated" with substrate at all these concentrations but as acetate was used up, the concentration in the M/80 vessels fell below the critical concentration for enzyme saturation.

TABLE 3

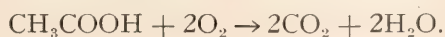
Effect of acetate concentration on oxygen consumption of logarithmic-phase cells
(Initial pH 5.8)

Time period	Control (inorganic buffer)	M/10 acetate	M/20 acetate	M/40 acetate	M/80 acetate
		<i>cu. mm. O₂/hour/million cells</i>			
0 to 60 min.....	5.72	39.28	55.50	62.36	64.27
60 to 120 min.....	4.42	44.46	61.40	67.52	69.12
120 to 180 min.....	4.01	46.96	64.88	68.39	19.23
180 to 240 min.....	3.37	48.62	66.58	69.56	4.80

Respiration in M/10 acetate was always markedly lower than in more dilute acetate concentrations (Table 3), although cell injury occurred in only one out of five experiments.

The growth phase of cells had a definite effect on their respiration in acetate. In numerous experiments it was found that in acetate medium, logarithmic-phase cells consumed from 40 to 60 cu. mm. O₂ per hour per million cells; while stationary-phase cells usually consumed from 20 to 30 cu. mm. O₂ per hour per million cells.

Experiments were performed to determine the respiratory quotient in acetate. In the CO₂ determinations bound carbon dioxide was liberated from the cell suspensions by tipping in 5N H₂SO₄ from the side-arms of the vessels (Dixon, 1934). The respiratory quotient in acetate was found to be approximately 1. (R. Q. values obtained in two experiments: 1.04, 1.03.) This value corresponds to the reaction for the complete oxidation of acetic acid to carbon dioxide and water:



The pH of cell suspensions in acetate usually increased somewhat (0.5 to 1.0 pH unit) during the course of an experiment.

No chemical tests were made to determine whether acetate was actually used up in the cell suspensions during these experiments. However, chemical analyses of old culture media have shown that the acetate concentration in cultures decreases with time (unpublished data). Hence it is reasonable to presume that the increase in respiration produced by acetate is due to the utilization and oxidation of this substance by the cells, rather than to some catalytic stimulating effect.

III. Tests for Cytochrome

The distribution of cytochrome among the Protozoa has received little attention; only a few records of its occurrence in members of this phylum have been published (cited by Jahn, 1941; Baker and Baumberger, 1941).

To determine whether cytochrome was present in *Astasia klebsii*, the usual method of spectroscopic examination was used. A very concentrated suspension of cells in acetate medium was placed in a small glass vial and examined by means of a Leitz micro-spectroscope. Three absorption bands were seen, located at approximately 605 $m\mu$, 565 $m\mu$, and 555 $m\mu$; these values agree fairly well with the generally reported locations of bands for cytochromes a, b, and c respectively (see Oppenheimer and Stern, 1939). Further evidence that these absorption bands were caused by cytochromes was furnished by the following observations:

(1) The bands disappeared after vigorous aeration of the suspension, then gradually reappeared and regained full intensity. (Aeration converts reduced cytochromes to oxidized cytochromes, which return to the reduced form when reducing conditions again prevail.)

(2) Addition of M/100 NaCN to the cell suspension did not change the absorption spectrum, but did prevent removal of the absorption bands by aeration. (Cyanide inhibits cytochrome oxidase, so that the cytochromes perforce remain in the reduced form.)

The oxidation of para-phenylene-diamine (abbreviated as PPD) has often been used as a test for the cytochrome-cytochrome oxidase complex, although this test is not wholly specific nor unambiguous (see Stotz, 1939, and Hogness, 1939). The effect of various concentrations of PPD upon oxygen consumption of *Astasia* was tested in several experiments. In a single instance M/200 PPD produced a 70 per cent increase in respiration and did not injure the cells; in all other experi-

ments, at PPD concentrations between M/20 and M/1000, the respiration was equal to or below the control respiration and nearly always extensive cell injury occurred.

IV. Effects of Respiratory Inhibitors

A. Cyanide

Cyanide is generally regarded as a useful tool for determining how much of the respiration of any organism is carried on through the cytochrome-cytochrome oxidase system, and has so been used in studies on numerous types of cells and tissues (review by Commoner, 1940). The spectroscopic detection of cytochrome in *Astasia klebsii* made it seem

TABLE 4

Effect of M/100 NaCN on the increase in respiration produced by various substrates at pH 5.8

Substrate tested	Exp. 1 Ethyl alcohol (M/20)	Exp. 2 Sodium formate (M/20)	Exp. 3 Sodium acetate (M/40)	Exp. 4 Sodium propionate (M/20)	Exp. 5 Sodium hexosediphosphate (M/40)
	cu. mm. O ₂ /hour/million cells				
Respiration in inorganic buffer before adding substrate (control).....	2.93	5.73	5.40	5.73	5.71
Respiration after adding substrate..... (% of control).....	8.15 (278%)	8.45 (147%)	41.43 (767%)	18.81 (328%)	8.41 (163%)
Respiration after adding M/100 cyanide to cell suspension in substrate..... (% of control).....	3.31 (113%)	3.35 (59%)	1.93 (36%)	3.60 (63%)	5.37 (94%)

likely that the cytochrome-cytochrome oxidase system played an important part in its respiration. Experiments on the effect of cyanide were accordingly undertaken.

Preliminary experiments were performed to determine the effect of cyanide on respiration in solutions of those substrates which had previously been found to increase oxygen consumption. It was found that the increase in respiration rate produced by formate, acetate, propionate, hexosediphosphate, and ethyl alcohol was usually completely abolished by M/100 sodium cyanide (Table 4).

Subsequently more detailed experiments on cyanide effect on respiration in inorganic buffer and in acetate medium were performed. The

cyanide solutions used were prepared by diluting M/1 NaCN with inorganic buffer of the same pH as the cell suspensions. The cyanide-KOH mixtures described by Krebs (1935) were employed as carbon dioxide-absorbing fluids, to prevent the escape of HCN from the cell suspensions during the course of an experiment.

Cyanide inhibition of respiration, both in inorganic buffer and in acetate, was found to be approximately the same at pH 5.8 and pH 7.9.

Cyanide inhibition of respiration is essentially a reversible reaction. It was necessary to show that cyanide did not visibly damage the cells, and that respiration went back to normal after removing the cyanide, before concluding that the only effect of cyanide was inactivation of the cytochrome-cytochrome oxidase system. Experiments along these lines were performed, employing cyanide solutions of various concentrations.

TABLE 5

Recovery of respiration from cyanide inhibition
(Logarithmic-phase cells in inorganic buffer, pH 5.8)

NaCN concentration	Respiration in cyanide (2 hours)	Recovery respiration (after removal of cyanide by washing) (2 hours)
	<i>cu. mm. O₂/hour/million cells</i>	
0	7.76	3.52
M/250	5.11	7.08
M/100	3.40	7.61
M/50	2.39*	5.20*

* Slight cell injury.

Using the erythrosin test, it was found that M/20 NaCN injured all astasias present within two hours. M/50 concentration usually injured a small percentage of the cells present. In M/100 cyanide there was scarcely any cell injury after four hours.

"Recovery" experiments were performed as follows: After a two-hour run in cyanide, the cell suspension in each Warburg vessel was removed and separately washed four times by centrifuging in inorganic buffer to remove the cyanide, then returned to the washed Warburg vessel for further observations on respiration. Results of a typical recovery experiment are shown in Table 5. These results show that when cells which had been in M/100 and M/250 cyanide were washed cyanide-free their rate of respiration rose to approximately the initial control level; for cells which had been in M/50 cyanide the recovery rate was somewhat below the initial control. Since M/50 cyanide proved somewhat toxic, it was not used in later experiments.

Figure 3 shows the results of a number of experiments on the effect of various concentrations of cyanide on respiration in inorganic buffer and in acetate, for logarithmic-phase cells and for stationary-phase cells. The experiments were performed at pH 5.8, using cyanide concentrations between M/100 and M/500,000.

For stationary-phase astasias under maximum cyanide inhibition (in M/100 cyanide), it was found that the cyanide-stable respiration was practically the same whether substrate was present or absent. In these

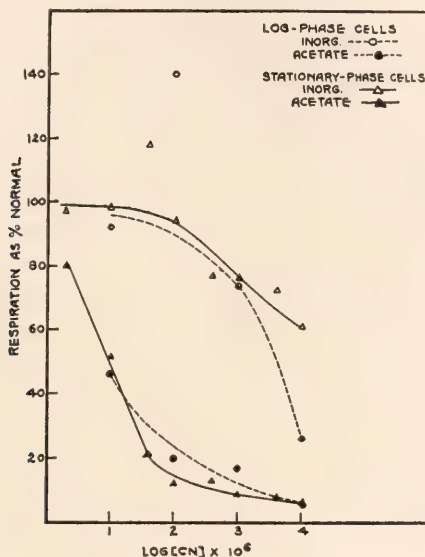


FIG. 3. Effect of different cyanide concentrations on respiration. Average values from three logarithmic-phase experiments and four stationary-phase experiments are represented (two-hour experiments at pH 5.8).

Normal respiration (average) as cu. mm. O₂/hour/million cells:

Log-phase cells in inorganic medium, 6.30

Log-phase cells in M/40 acetate, 46.96

Stationary-phase cells in inorganic medium, 2.28

Stationary-phase cells in M/40 acetate, 24.63

experiments, the average cyanide-stable respiration (expressed as cu. mm. O₂/hour/million cells) was 1.37 in inorganic buffer and 1.46 in acetate. In inorganic medium the total (cyanide-free) respiration was small, so that the percentage of inhibition by M/100 cyanide was relatively slight. But when substrate in sufficient concentration (M/40 sodium acetate) was present the cyanide-sensitive system presumably functioned at full capacity, a high total (cyanide-free) respiration was attained, of which the cyanide-stable portion formed only a small frac-

tion—consequently over 90 per cent of the respiration of substrate-saturated cells was cyanide-sensitive. For logarithmic-phase cells in M/100 cyanide the situation was much the same. The foregoing is in agreement with Commoner's (1940) statement: "Since most variations in the total rate of respiration are mainly due to variations in the activity of the cyanide-sensitive system alone, percentage inhibition by cyanide (i.e. "cyanide-sensitivity") increases with the normal rate of respiration."

Cyanide at high concentration (M/100 and M/1000) produced a marked decrease in the amount of paramylum in the cells in long-term experiments. It seems possible that this phenomenon may have been due to the effect of cyanide in increasing activity of amylase, thus resulting in more rapid breakdown of paramylum. The effect of cyanide in stimulating amylase activity under certain conditions has been demonstrated by several workers (Hanes and Barker, 1931; Denny, 1931).

B. Azide

Keilin (1936) studied the inhibition of cellular respiration by sodium azide (NaN_3), and showed that its mode of action was in general similar to that of cyanide. However, he and later workers pointed out certain

TABLE 6
Effect of pH on azide inhibition of respiration of stationary-phase cells

NaN ₃ concentration	At pH 5.8		At pH 7.9	
	Inorganic buffer	M/20 acetate	Inorganic buffer	M/20 acetate
	<i>cu. mm. O₂/hour/10⁶ cells</i>		<i>cu. mm. O₂/hour/10⁶ cells</i>	
0 (Control).....	1.92	26.51	1.96	26.52
M/1000.....	2.90		2.31	
M/500.....		1.26		26.23
Azide inhibition.....	(Up 51%)	(95%)	(Up 18%)	(1%)

(No cell injury occurred in this experiment.)

interesting differences in the effects of these two substances. In view of these facts a study of azide effects was included in the present investigation.

The relation between pH and azide inhibition of respiration in *Astasia* was investigated (Table 6). In acetate medium, M/500 azide had no effect at pH 7.9, but produced 95 per cent inhibition of respiration at pH 5.8. In inorganic buffer there was no azide inhibition at either pH level.

Somewhat similar relations between pH and azide inhibition have been reported by Keilin (1936), who studied the effect of azide on respiration of yeast cells, and by Armstrong and Fisher (1940), who studied the effect of azide on frequency of the embryonic fish heart. Pointing out that at low pH much of the azide would be in the form of

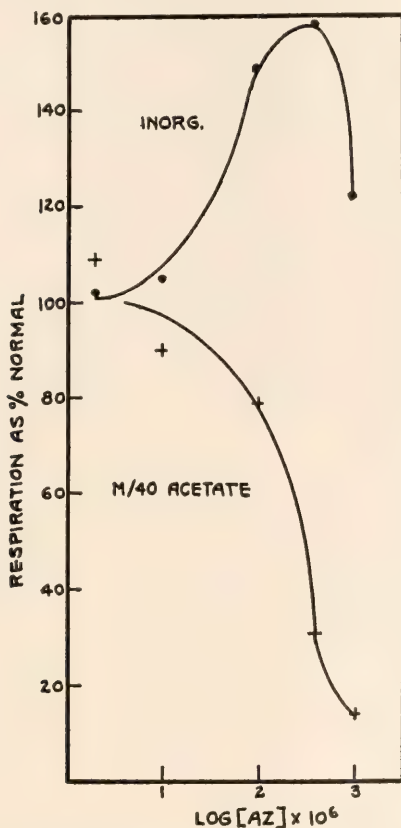


FIG. 4. Effect of different azide concentrations on respiration at pH 5.8. Average values from six two-hour experiments, using cells in logarithmic and stationary phases of growth.

undissociated hydrazoic acid, Armstrong and Fisher concluded that only in this undissociated form could azide enter fish embryo cells. On the other hand, Stannard (1939) found no such pH effect involved in azide inhibition of respiration of active frog muscle.

Experiments were performed at pH 5.8 to determine the toxic level of azide concentration. It was found that in M/250 azide nearly all the cells were injured within two hours (five out of five experiments).

M/500 azide usually injured a large percentage of the cells present (seven out of eight experiments). In M/1000 azide no cell injury was observed in most cases (10 out of 12 experiments). Azide was thus toxic to the cells at much lower concentrations than cyanide.

The effect of azide concentrations between M/500,000 and M/1000 on respiration at pH 5.8 was investigated. Results are shown in Figure 4. In acetate medium, an inhibition curve more or less resembling that produced by cyanide was obtained. But in inorganic buffer, no azide inhibition occurred at any concentration tested, while at certain azide concentrations there was a pronounced increase in respiration over the control values.

Stannard (1939) suggested that in frog muscle there appear to be two distinct respiratory mechanisms: one is characteristic of resting muscle and is only cyanide sensitive; the other functions only in activity, includes the cytochrome system, and is both azide and cyanide sensitive. Similarly, in *Astasia*, it appears that respiration in inorganic medium is mediated through a cyanide sensitive but azide insensitive system; while respiration in acetate takes place through a system which is both azide and cyanide sensitive.

SUMMARY

1. Concentrated cell suspensions of *Astasia klebsii* were prepared from pure cultures. The respiration of these suspensions was measured by the Warburg direct method at 25.2° C., usually at pH 5.8.

2. Cells in inorganic medium showed an early period of declining respiration, followed by an approximately constant period. During the constant period, old (stationary-phase) cells consumed about 1.9 cu. mm. O₂/hour/million cells, and young (logarithmic-phase) cells consumed about 3.8 cu. mm. O₂/hour/million cells.

3. The effects of numerous organic compounds on respiration were tested. Only formate, acetate, propionate, ethyl alcohol, and hexosediphosphate produced a definite increase in oxygen consumption, the greatest increase occurring in acetate.

4. In inorganic medium, respiration was approximately the same between pH 4.5 and pH 7.9. In acetate, respiration was the same at pH 5.8 and pH 7.9, and much lower at pH 4.5.

5. In acetate the respiratory quotient was 1.0; in inorganic medium it was slightly less than 1.0.

6. The presence of cytochrome in the cells was detected by spectroscopic examination.

7. The highest non-toxic concentration of cyanide (M/100) produced about 95 per cent inhibition of respiration in acetate, and a lesser degree of inhibition in inorganic medium. The highest non-toxic concentration of azide (M/1000) inhibited nearly 90 per cent of the respiration in acetate, but had no inhibitory effect on respiration in inorganic medium.

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THE ACCOMMODATION OF SOME MARINE INVERTEBRATES TO REDUCED OSMOTIC PRESSURES

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It has been demonstrated by Pearse (1927), Schlieper (1935), Beadle (1931), Sayles (1935) and others that marine forms will survive dilutions of sea water greater than those encountered in their natural environment for various lengths of time. That this ability will be of importance in determining the degree to which a marine species will colonize areas subject to fluctuations in salinity seems likely, but the precise degree of correlation has been little studied. The work reported here consists of two parts: (1) a survey of the distribution of some marine invertebrates in the Narraguagus River estuary, and (2) an experimental investigation on the tolerance to reduced osmotic pressures of the same forms in the laboratory. The entire study was necessarily confined within the period from February, 1940 through April, 1941. The Narraguagus River estuary is located in the town of Millbridge on the coast of Maine. The ecological study consisted of the location of marine species along the estuary, and a study of the freezing point of the water, water temperature, and type of bottom at selected stations.

The experimental work consisted of (1) the determination of the ability of 14 species to live in diluted sea water, (2) a comparative study of the resistance to diluted sea water of specimens of the same species collected from brackish water and from the open sea, and (3) the influence of diluted sea water upon the weight and oxygen consumption of *Nereis virens*. Finally an attempt has been made to correlate the results of the three investigations in an effort to construct conclusions of ecological importance.

METHODS AND OBSERVATIONS

The Narraguagus River is relatively large near its mouth and is about 300 feet wide at the town of Millbridge. The distance from the open sea to the head of the tide water is approximately ten miles. The

¹ Based upon a thesis presented by the senior author in partial fulfillment of the requirement for the degree of Master of Arts.

widening bay into which the river empties is hemmed in by numerous islands and mainland points, and at low tide vast areas of exposed mud flats, ledges, and rocky shores furnish an ideal environment for many common marine invertebrates. With such a body of fresh water entering the sea, it seemed reasonable to assume that there would be regular changes in the salt concentration with the tides, and that sessile forms of life would be subjected to rapid changes in the osmotic pressure of the external medium in their natural environment.

Freezing points were measured in a simple cryoscope by means of a Heidenhain thermometer. Samples were collected at 15 stations (see



FIG. 1. Map of the lower portion of the Narraguagus River and of Narraguagus Bay, showing points of collections. Station one is at head tide. Stippled areas represent mud flats exposed at low tide. Depths are at mean low tide. Average tide, 11 feet. After U. S. G. S. Cherryfield and Petit Manan quadrangles.

Fig. 1) in small air-tight jars having a capacity of ten cubic centimeters, and were tested in the laboratory after filtering within 48 hours. As can be seen in Table I, the freezing point values at high tide are lower than those at low tide at all points except station one. In general there is a greater depression of the freezing point towards the open sea, although some irregularities appear because of the contours of the channel.

TABLE I

Results of preliminary investigation of Narraguagus River estuary and location of marine species in this region

Station	Freezing point °C.			Temperature		Marine species
	High tide	Low tide	Water extracted from mud	High tide	Low tide	
1	-.011	-.011		21	21	
2	-.021	-.014		19	20.5	
3	-.141	-.116		18.2	20	<i>Balanus balanoides</i>
4	-.948	-.145		17.5	19.6	<i>Grammarus</i> sp. <i>Crango septemspinosus</i> <i>Nereis virens</i>
5	-1.006	-.404		17.2	19.2	<i>Mya arenaria</i> <i>Mytilus edulis</i> <i>Electra pilosa</i> <i>Flustrella hispida</i> <i>Lepidonotus squamatus</i> <i>Spirorbis spirorbis</i> <i>Acmaea testudinalis</i> <i>Littorina litorea</i> <i>Thais lapillus</i> <i>Modiolus modiolus</i>
6	-1.014	-.537	-1.345			<i>Balanus balanus</i> <i>Balanus eburneus</i> <i>Jaera marina</i>
7	-.978	-.438	-1.702			
8		-1.120	-1.386			<i>Sertularia</i> <i>Hippothoa hyalina</i> <i>Cerebratulus lacteus</i> <i>Tegella unicornis</i> <i>Glycera dibranchiata</i>
9		-1.212	-1.828			<i>Orchestis platensis</i> <i>Nucula proxima</i>
10	-1.624	-1.236	-1.622			
11		-1.615	-1.638			
12	-1.756	-1.374				<i>Lineus ruber</i> <i>Harmothoe imbricata</i> <i>Nephtys caeca</i> <i>Clymenella torquata</i>
13	-1.791	-1.491				<i>Asterias vulgaris</i>
14		-1.706	-1.717			<i>Strongylocentrotus drobachiensis</i>
15		-1.771				
16	-1.791	-1.791				<i>Metridium dianthus</i> <i>Tealia crassicornis</i>
18	-1.791	-1.791				<i>Bunodes stella</i> <i>Clava leptostyla</i>

The osmotic pressure of the water confined within the mud flats was investigated, since the animals living in the mud are in contact with this confined water rather than the free surface water. A special type of apparatus (Fig. 2) was designed to extract mud at depths optimal for marine worms without surface contamination. A small portion of each end of the core was discarded and the remainder placed in air-tight jars for transportation to the laboratory. Water was extracted by centrifugation and its freezing point was determined. The tubes were covered during this process to prevent evaporation.

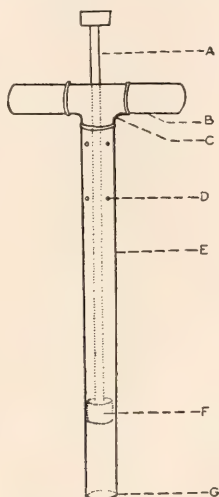


FIG. 2. Mud sampler. A. Plunger rod; B. Wood handle; C. Brass T pipe; D. Air expulsion holes; E. 1" brass pipe; F. Rubber plunger; G. Beveled cutting edge.

The freezing points of this "confined" water from all stations show only slight variations, and differ from those of the surface water in the fact that they do not change much in relation to the distance of the station from the open sea. The variations found may be attributed to differences in the physical qualities of the mud at the various stations, and to the proximity of the station to the river channel. It is notable that in every case the osmotic pressure of the confined water is greater than that of the surface water at high tide. This difference is greatest towards the head of the tide water. It is evident that animals burrowing into mud will not be exposed to the rapid fluctuations in osmotic pressure to which animals attached to the surface are exposed.

At each station the marine species present were collected and identified. In Table I each species is listed under the station farthest up

the estuary at which it occurred. It is to be understood that all species were also found at stations nearer the open sea and in greater numbers in these situations. In this work the open sea was considered to be that region at station 16 and beyond where no tidal variation in the freezing point of the water was observed. Particularly noticeable in the low salinity area was the absence of animals belonging to the phyla Porifera, Coelenterata (with the exception of Sertularia), and Echinodermata. However representatives of these phyla were extremely abundant beyond station 15. Another outstanding fact was the size of the animals at station four which were much smaller than similar forms in regions of higher salinity. The worm *Nereis virens* was only two to two and one-half inches long at station four, while it averaged eight inches in the bay proper.

Experiments on Acclimatization to Low Salinity

The experimental animals were brought in from the field and placed in glass dishes of 350 cc. capacity. The dishes were placed in a cooling bath maintained at about 15° C. by means of circulating water. Instead of changing the water in the dishes frequently as other workers have done, air was bubbled continuously through porous "air stones." The entire bath was covered by a glass plate to keep evaporation of the solutions at a minimum. Clean sea water having a freezing point of —1.791° C. was collected from the open sea at station 16. Dilutions were made with fresh water taken from the Narraguagus River above the dam at station one. After ice formed in the river it was supplanted by Orono tap water. The experiments were started in June, 1940, at Millbridge, and were transferred to the University of Maine laboratories in September. Three series of experiments were carried out.

Opportunity for Acclimatization and Survival of Nereis virens in Fresh Water

Twelve worms of the same size and weight were paired and placed in six dishes containing sea water. Worms were carried down to fresh river water as follows: *A*, from sea water directly into fresh water; *B*, from sea water to 50 per cent sea water to fresh water; *C*, through dilution intervals of 25 per cent; *D*, through dilution intervals of 10 per cent; *E*, through dilution intervals of 5 per cent and *F*, through dilution intervals of 1 per cent. All dilution intervals are stated on the basis of the original concentration, and were made either by adding a calculated amount of fresh water, or by changing the entire medium when the capacity of the dish was reached. A period of 24 hours was allowed

for accommodation to each new dilution in groups *A* to *E*. In group *F* the accommodation period was shortened to eight hours, since this period proved long enough to permit adjustment to small alterations in concentration. Control animals were kept in sea water during the entire experiment. The results are given in Table II.

TABLE II

Effects of varying rate of dilution of the external medium on the survival time in fresh water of Nereis virens

Group	Survived 24 hours in each of the following concentrations of sea water	Average survival time in fresh water: hours
A	100 (to fresh water)	6
B	100, 50 (to fresh water)	19
C	100, 75, 50, 25 (to fresh water)	22
D	100, 90, 80 . . . 20, 10 (to fresh water)	32
E	100, 95, 90 . . . 10, 5 (to fresh water)	61
	Survived 8 hours in the following concentrations of sea water	Average survival time in 2% sea water
F	100, 99, 98 . . . 3.2*	72

* Limit of survival in this experiment.

It will be noted that *Nereis* was able to survive in fresh water for longer periods of time as the acclimatization process was made more gradual. Although the animals in this experiment were not weighed, the increased body size and turgidity of the worms due to water intake was very evident in the cases where the dilution interval was large. This turgid condition was less noticeable as the dilution interval became smaller. In group *E* it was not observed until the worms had passed below the 25 per cent sea water level. Subsequently group *F* reached 10 per cent sea water before this condition became apparent. This would indicate that *Nereis* can accommodate to reduced osmotic pressure if the change is gradual enough. It should be noted that the worms in group *F* died in 2 per cent sea water after surviving in that concentration for 72 hours. Work was necessarily suspended at that time so that the survival time in fresh water could not actually be ascertained. At very low salinities frequent changes of the water were necessary, even with a liberal amount of air being supplied. This was due to what seemed to be an excessive expulsion of coelomic fluid which caused putrefaction of the medium. There is a possibility that this fluid was eliminated through the nephridia. The control animals were in good condition when the experiment was discontinued 42 days after it was started.

*The Comparative Susceptibility of Fourteen Species to Reduced
Osmotic Pressure*

Fourteen species representing the phyla Coelenterata, Nemertea, Annelida, Arthropoda, Mollusca and Echinodermata were selected because of their availability in the bay. The apparatus and methods were the same as those described above. Dilutions were made through 1 per cent intervals every eight hours until the animals appeared to have reached their critical point for survival, as indicated by sluggishness and turgidity. The time between changes was then increased to 72 hours. The number of animals in each dish varied between one and 25 depending upon the size. In most cases only two or three were confined in the same dish.

Table III records the results of this investigation. The freezing point values listed are those of the water at low tide at the station where the animals were collected. These areas were selected for collecting specimens because of the numbers available. All animals were in excellent condition when brought to the laboratory with the exception of *Tealia crassicornis* which could not be removed from the rocks without injury to the pedal disc. It should be stated too that it was very difficult to keep *Asterias vulgaris* for any length of time even in natural sea water. The control animals lived very little longer than the experimentals. It is probable that their brief survival was due to other factors. One such factor was nutrition. Even though whole clams and clear chopped clam tissues were placed with them, they failed to eat.

There was considerable variation among individuals of the same species in their abilities to survive in low concentrations of sea water. In a few instances the difference between the minimum and maximum lethal concentrations is high (e.g. *Mya arenaria*), but in most cases the difference is 10 per cent or less. In general it was found that the smaller animals of each species were better able to survive than the larger members. Four *Nereis virens* lived in 1 per cent sea water 52 hours, and three *Gammarus* lived in fresh water for 72 hours. *Glycera dibranchiata* became very swollen and turgid in 50 per cent sea water, and in greater dilutions the integument in certain body regions burst causing extensive loss of blood. *Jaera marina* survived in fresh water for two weeks, and were discarded to make room for other experiments.

On the basis of these results a general phyletic relationship can be established in regard to the ability of animals to survive in reduced salt concentrations. Such a classification stated in order from greatest to

least ability of survival is: Arthropoda, Annelida, Mollusca, Nemertea, Echinodermata and Coelenterata. With the exception of Coelenterata, this order conforms roughly to the degree to which these phyla have succeeded in colonizing fresh water.

TABLE III

The comparative abilities of fourteen species to withstand reduced osmotic pressures arranged in order of decreased resistance

Animal	Freezing point of water in collecting areas	Number used	Per cent sea water withstood -1.791°C.		
			Min. ¹	Max. ²	Average
<i>Jaera marina</i>	-1.791	25	..	..	0
<i>Gammarus</i> sp.....	-1.212	18	0.5	10	2
<i>Nereis virens</i>	-1.120	12	2	2	2
<i>Crago septemspinosus</i>	-0.438	4	3	5	4
<i>Balanus balanoides</i>	-1.212	4	7	13	9
<i>Mytilus edulis</i>	-1.212	8	2	25	9.6
<i>Thais lapillus</i>	-1.212	6	6	24	13
<i>Balanus balanus</i>	-1.212	3	10	18	14
<i>Glycera dibranchiata</i>	-1.212	8	30	38	35
<i>Mya arenaria</i>	-1.120	6	8	55	36
<i>Cerebratulus lacteus</i>	-1.120	6	42	58	47
<i>Asterias vulgaris</i>	-1.791	6	66	84	73
<i>Clava leptostyla</i>	-1.791	2 col.	71	76	74
<i>Tealia crassicornis</i>	-1.791	6	80	90	85

¹ Minimum percentage concentration below which no animals survived for 72 hours.

² Maximum percentage concentration above which no animals died within a 72-hour period.

A Comparison of Individuals from the Open Sea and from Brackish Water in Their Resistance to Diluted Sea Water

In this experiment members of four species, collected from areas exposed to the open sea (station 18), were carried through a procedure as described in the preceding section. Their survival was compared with that of brackish water forms collected from station seven. The results of this experiment are shown in the upper part of Table IV with results extracted from Table III added so that an effective comparison can be made. It will be seen readily that in every case the open sea individuals were less resistant to changes in the concentration of sea water than the brackish water forms. The difference is less pronounced in some species, and suggests a higher development of the osmotic regulatory mechanism in these species. The comparison may be made still

more significant by the fact that after open sea specimens of *Mya* had succumbed, brackish water clams introduced into the same concentration of sea water survived and were able to adjust down to a limit of 10 to 8 per cent sea water.

TABLE IV

The comparative abilities of similar open sea and brackish water forms to withstand reduced osmotic pressures

Animals from open sea areas $\Delta = -1.791$	Number used	Dilution per cent sea water		
		Min.	Max.	Average
<i>Nereis virens</i>	6	6	8	7
<i>Gammarus</i> sp.....	9	3	16	8
<i>Mytilus edulis</i>	5	39	46	43
<i>Mya arenaria</i>	8	48	58	52
Animals from brackish water $\Delta = -.978$				
<i>Nereis virens</i>	12	2	2	2
<i>Gammarus</i> sp.....	18	0.5	10	2
<i>Mytilus edulis</i>	8	2	25	9.6
<i>Mya arenaria</i>	6	8	55	36

Effects of Gradually Reduced Osmotic Pressure on the Weight and Oxygen Consumption of Nereis virens

According to Beadle (1931) *Nereis diversicolor* increases in weight on transfer to dilute sea water and also increases its respiratory rate. Sayles (1935) likewise found an increase in the weight of *Nereis virens* in dilute sea water, but did not determine the respiratory rate. In their experiments the worms were transferred directly to water of low salinity. In the experiment here described five animals were transferred daily through 4 per cent dilution intervals. The Rideal-Stewart modification of the Winkler method was used to determine the amount of dissolved oxygen in the water in closed vessels before the worms were placed in them, and after they were removed. The worms were kept in the closed containers four hours, during which period the apparatus was darkened so as to keep activity at a minimum. Figure 3 shows the results of this experiment. The average weight of the worms shows a slight but consistent decrease even down to 50 per cent sea water. Then there is a marked increase in weight at 28 per cent sea water, but a change to still more gradual dilutions brought the weights down again.

The final weight rise beginning at 16 per cent appeared to be due to the breakdown of the osmoregulatory mechanisms, and was characterized by the intake of large amounts of water which made the animals turgid. Up to this point the animals remained firm with no sign of excess water either in the coelom or the integument.

The results of the oxygen consumption measurements show much more variation between individual worms. This is probably due to differences in the amount of activity. A rather definite trend of the averages is evident, however, and this is correlated with the weight

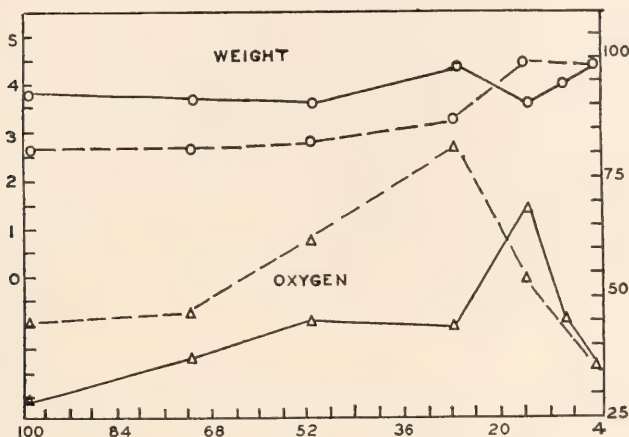


FIG. 3. Effects of diluted sea water on the weight and oxygen consumption of *Nereis virens*. Left hand ordinates represent total weight of five worms in grams; right hand ordinates represent average cu. mm. oxygen per gram per hour. Abscissa; percentage concentration of sea water. Continuous line represents readings as animals were carried from 100 per cent sea water to 4 per cent. Dashed line represents the reverse experiment.

curve. With moderate dilutions the weight remained constant, but oxygen consumption rose 33 per cent. In the experiment described below a corresponding but much sharper decrease in oxygen consumption was found as the worms were returned to normal sea water. We believe that the increased oxygen consumption in dilute sea water represents the expenditure of energy for osmotic work. Below the critical range of 28 to 16 per cent sea water there was a decrease in the amount of oxygen used per gram per hour. Since weight was increasing during this period it is difficult to separate the decrease due to the intake of metabolically inactive water from that which may be expended in osmoregulation.

Weight and Oxygen Consumption of Acclimatized Nereis virens when Gradually Returned to Natural Sea Water

The same animals used in the experiment described above were carried from 4 per cent sea water back to natural sea water, but necessarily at a more rapid rate. Tests of oxygen consumption were made at the same concentrations as in the previous experiment except at 10 per cent sea water. Apparently osmotic regulation is restored as the osmotic pressure of the medium is increased. This is indicated by the sudden decrease in weight accompanied by a marked increase in oxygen consumption up to the critical point at 28 per cent sea water. From that concentration up to full strength sea water there is a direct relationship between the change in weight and the amount of oxygen consumed for, as there is less need for regulation, less energy is expended by the animals. Particularly noticeable at the end of this experiment was the fact that the average weight of the worms had declined 38.5 per cent from the original value. Some of this loss may be due to loss of salts, but since the animals were used experimentally over a period of 94 days, it is probable that other features of an artificial environment may have contributed to this condition.

DISCUSSION

Experimentally all brackish water animals except *Balanus balanoides* were able to survive in media of lower osmotic pressure than those encountered in their natural environment. It may be that the eggs or juvenile stages are less resistant to dilution of the sea water, so that the species fails to invade the less saline areas. It is also possible that the animals avoid water below a certain salt concentration even though they can live in it. In the Narraguagus a complication is found in the presence of accumulations of sawdust from mills located on the river. This is abundant as far south as station four, and undoubtedly has an unfavorable effect on many marine organisms.

According to Adolph (1925) most marine animals are able to live after abrupt change to almost pure fresh water provided that the remaining salts are present in physiological proportions, and gradual dilutions over several days do not materially help the animals to endure pure water. Although he states that some species lived in 1 per cent sea water he names no specific organisms. This is in disagreement with the results of other workers. Pearse (1927) was able to carry few species into solutions weaker than one-fourth sea water, while most of the 18 forms he worked with could not survive in concentrations less

than 50 per cent sea water. Sayles (1935) found the minimum survival concentration for *Nereis virens* to be between 30 and 40 per cent sea water. In these experiments it has been possible to obtain better results than some other workers by keeping the animals at a relatively constant temperature approximating that of their natural environment, maintaining constant aeration, and preparing dilutions with fresh water collected from the Narraguagus River. Dilutions made with distilled water were found to be definitely toxic, while Orono tap water did not give as good results as river water.

Other work bearing on the effect of salinity changes upon respiration appears to be scarce. Beadle (1931) reports that Turussov (1927) found by carbon dioxide measurements with a modified Osterhout apparatus that the respiratory rate of *Nereis diversicolor* increased in hypotonic and decreased in hypertonic sea water. Beadle himself and Schlieper (1935) confirmed these results, but Krogh (1939) states that the small increase in oxygen consumption shown in their calculations does not exceed the limits of error, and therefore cannot be regarded as significant. He admits that energy must be expended for osmotic work, but claims that the amount is too small for demonstration. In view of the unknown efficiency of this process, however, the prediction may not be valid.

Both Beadle and Sayles report that the weights of several *Nereis diversicolor* and *Nereis virens* paralleled one another for a time, but that some would soon decrease in weight at the same time that others were increasing. In many cases those animals that continued to increase in weight became turgid with a resulting rupture of the body wall allowing considerable loss of body fluid. The weights of *Nereis virens* in the experiments here reported showed very little variation, and then only in the extremely low salinities, while no animals were observed to become swollen to the bursting point. *Glycera dibranchiata*, on the other hand, took on large amounts of water in concentrations of 50 per cent sea water and in 39 per cent sea water the integument burst with considerable loss of blood and internal fluids.

A comparison of the relative ability of the three species of *Nereis* which have been tested is difficult because of differences in technique. *Nereis virens* appears to use less oxygen per gram weight than do the other two species, but on the basis of surface area the amounts consumed are about the same.

SUMMARY

1. The distribution of 14 species of invertebrates in the Narraguagus Bay and River was compared with the osmotic pressure (indirectly de-

terminated by means of freezing points) of the water at a series of stations ranging from the open sea to the head of the tide.

2. The osmotic pressure of the water confined in the mud flats was found to be almost constant at high and low tides in contrast with the marked tidal variations in surface water.

3. Typical marine animals were found in an environment having a freezing point of $- .404^{\circ}$ C., or the equivalent of 19 per cent sea water.

4. The survival of *Nereis virens* in fresh water was extended as the process of acclimatization was made more and more gradual.

5. Tests on 14 species showed that all but one were able to survive experimental exposure to reduced osmotic pressure below that found in their natural environment.

6. Members of a species collected from water of low salinity were more resistant to reduced osmotic pressure than individuals collected from the open sea.

7. *Nereis virens* was able to accommodate without marked change of weight down to a concentration of 16 per cent sea water. At this point a rapid increase in weight occurred. The dilution process above 16 per cent was accompanied by an increased oxygen consumption. Below 16 per cent sea water oxygen consumption decreased.

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INCREASE OF CORTICAL CALCIUM WITH AGE IN THE CELLS OF *ELODEA CANADENSIS*¹

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The suggestion has been made frequently that senescence results from progressive accumulation in the cell of materials which either are toxic or obstruct metabolism (Jickeli, 1902; Montgomery, 1906; Child, 1915; Seifritz, 1936; Heilbrunn, 1937). Benedict (1915) formulated the hypothesis that senescence is a result of a decrease in the permeability of cells. Both of these causes of senescence may be the result of an increase with age in the calcium content of the cell membrane, for calcium decreases the permeability of cells to at least some classes of substances (see Heilbrunn, 1937, for literature review). Thus it is conceivable that an increase in the calcium content of the cell cortex could decrease the cellular permeability to its toxic metabolic waste products as well as other substances. A gradual accumulation of these substances in the cell may be a consequence of these preceding changes, and singly or in combination may produce the degenerative changes of senescence. In view of the universality of senescence the author inclines towards the hypothesis that the single or series of related cellular changes sketched above is its universal cause. He proposes therefore to present a series of studies on the possible localized increase of calcium with age in a variety of organisms, and its consequences. It should be noted that Molisch (1938) suggested a relation between the deposition of calcium with age in cell membranes of plants and a decrease in the permeability of cells.

CALCIUM CHANGES WITH AGE

Increase of calcium with age is one of the commonest features of the aging process. The calcium content of many human tissues and organs has been shown to be greater in old individuals than in young individuals

¹ Part of the dissertation submitted to the faculty members of the graduate school in partial fulfillment of the requirements for the degree, doctor of philosophy, in the Department of Zoology, Indiana University. Contribution No. 297 from the Zoology Laboratory, Indiana University.

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(Burger and Schlomka, 1927; Delbet and Bretau, 1930; Hesse, 1934; Sorsby, Wilcox and Ham, 1935; and Simms and Stolman, 1937).

The work of Novi (1913) on other mammals is in agreement with the findings in human material; he reports that the calcium content of the brain of the guinea pig at birth is very low, but toward the end of the life span it increases markedly. Similarly, Lansing (unpublished observations) found a progressive increase in the calcium content of the rat brain with age. On the other hand, Novi (1913) found that the calcium content of the dog brain is very high in the fetus, is very low in the young animal and finally returns to the high level of the fetus in the old dog. Sorsby, Wilcox and Ham (1935) reported that the amount of calcium in the sclera of the cat eye decreases from birth to adult life, then increases with old age. Lastly, Cahane (1927) concluded that the amount of calcium per gram of fresh muscle of the guinea pig, dog, cat and rabbit decreases with age. It is apparent from Cahane's data, however, that the majority of animals referred to as "old" were actually either immature or young adult animals.

The preceding studies, with the exception of the one by Cahane (1927), demonstrate that the calcium content of mammalian tissues increases after maturity. It is also apparent that the calcium content of fetal tissues may be very high, and that the calcium content decreases in some animals during the period of immaturity.

As far as the writer has been able to determine, there have been no studies of calcium changes with age in plants or invertebrates. The observation that the amount of insoluble salts of calcium increases with age in plants is a common one but has not been put on a quantitative basis. Molisch (1938) points out that calcium oxalate and carbonate are precipitated about the cell walls of plants and increase with age. He suggests the possibility that calcium increases with age in the cell membrane but offers no evidence to support this view. The writer has undertaken a study of calcium changes with age in plants and the invertebrates. This paper deals with the water plant, *Elodea*; a later paper will deal with the calcium changes with age in the invertebrates *Euchlanis*, a rotifer, and *Phagocata*, a planarian.

Mazia and Clark (1936) studying the effects of various stimulating agents on *Elodea* leaves found that upon stimulation calcium is released from the cortex of the cell and combines with the soluble oxalates in the central cell vacuole to form the insoluble crystals of calcium oxalate. The technique of Mazia and Clark suggests a means of determining the amount of calcium in the cortex of *Elodea* cells of varying ages. The following experiments were undertaken to investigate this possibility.

MATERIALS AND METHODS

Freshly cut stalks of *Elodea canadensis* (*Anachris canadensis*) grown in a glass aquarium at room temperature were employed in all the experiments. The stalks used were a minimum of six inches in length and a maximum of ten inches.

The relative age of an *Elodea* leaf is easy to determine by the position of the leaf on the stalk. Young leaves are located at the apex of the stalk and old leaves are located at the base of the stalk.

The technique used for the study of the calcium content of *Elodea* cells was based upon the observation by Mazia and Clark (1936) that stimulation of the *Elodea* cell results in the combination of the calcium in the cortex of the cell with the oxalates in the vacuole to form insoluble crystals of calcium oxalate. Stimulation was electrical in the present experiments.

Leaves were removed for analysis from various parts of the stalk and were placed on glass slides. Two $1\frac{1}{2}$ volt dry cells were connected in series with an induction coil set for interrupted current. Platinum electrodes were placed directly on the moist leaf and the electrodes were reversed immediately upon the expiration of one half of the total time of stimulation which was two minutes.

Tests were conducted to determine the amount of stimulation required to produce maximal formation of calcium oxalate crystals in the cells. It was found that stimulation for two minutes was adequate to produce the maximal effect in all cases. A young leaf was stimulated for 40 seconds. Thirty-five crystals per cell were formed and subsequent stimulation produced no increase in the number of crystals. Stimulation of an old leaf for 40 seconds produced 15 to 20 crystals, stimulation for 60 seconds produced 30 to 40 crystals, while stimulation for 80 seconds produced 50 to 55 crystals per cell. Further stimulation produced no increase in the number of crystals formed. This strongly suggests that old cells are less susceptible to stimulation than young cells, or rather that they are less irritable; however, no studies were conducted to verify this point.

The description of the calcium oxalate crystals furnished by Mazia and Clark (1936) was used as a basis of identification of the monoclinic and tetragonal crystals found in these experiments. Five small and large tetragonal and large monoclinic crystals, and 46 small monoclinic crystals (in apical and basal leaves) were selected at random for measurement. The lengths and widths were measured by means of a micrometer ocular and the depths by the calibrated fine adjustment screw of the microscope. The depth measurements involve an error of ± 0.5



micron. Small monoclinics average 0.9 ± 0.08 micron in width, 2.5 ± 0.09 micron in length and 1.0 micron in depth. Small tetragonal crystals average 3.3 ± 0.08 micra in width, 3.3 ± 0.05 micra in length and measure 2.0 micra from apex to apex of the crystal. Large tetragonal crystals average 6.8 ± 0.21 micra in width, 7.2 ± 0.00 micra in length and 4.5 micra from apex to apex of the crystal. Large monoclinic crystals average 3.3 ± 0.25 micra in width, 6.2 ± 0.32 micra in length and 3.0 micra in depth.

The volumes of the various types of crystals were computed on the basis of the above measurements. As the monoclinic crystals are rectangular parallelepipeds, the product of the three linear dimensions of the monoclinic crystals was employed for the volume calculation. Since the tetragonal crystal is essentially two pyramids whose bases are in juxtaposition the formula for computing its volume is two times the area of the base times the height of the pyramid divided by three, i.e., twice the volume of a pyramid. Thus the average volume of the small monoclinic crystals is 2.2 ± 0.33 cubic micra, small tetragonal is 21.5 ± 0.7 cubic micra, large tetragonal is 222.3 ± 13.4 cubic micra and large tetragonal crystal is 62.6 ± 7.0 cubic micra. The calcium content of the cells studied was determined by making counts of the number of crystals present after stimulation and computing the total volume of crystalline material.

Since the amount of crystalline material formed in these experiments could possibly be correlated with the amount of cell surface present as well as the concentration of calcium in the cell cortex, it was necessary to determine the total amount of surface for the various cells.

Measurements with a micrometer ocular were made of six cells in the tip region of young and of old leaves. The sum of the areas of the surfaces of the cell was used in the computation of the total cell surface. The total surface of the mean apical cell in apical leaves was 2919 square micra and of the mean apical cell of basal leaves was 3139 square micra. Thus, the total cell surface of apical cells of basal leaves was only seven per cent greater than that of apical cells of apical leaves.

Quantitative determinations of the oxalate content of apical regions of young leaves were made. For each determination 75 leaves were used; the anterior fifth of each leaf was cut off and put in a porcelain crucible containing distilled water. The material was thoroughly crushed and the supernatant fluid decanted. The residue was washed several times with distilled water and the washings added to the material previously removed. The remaining pulp was filtered and washed, the filtrate again being added to the previous wash solutions. The solution was rendered alkaline with NH_4OH and 2 cc. $\text{N}/3 \text{ CaCl}_2$ was added in

order to obtain complete precipitation of the oxalate. The solution was then filtered and the residue dissolved in 15 cc. of hot H_2SO_4 (1:5). The filter paper was washed with an additional 15 cc. of H_2SO_4 at 70°C ., and the filtrate was added to the solution previously obtained. The solution was kept at 70°C . and titrated against 0.01 N KMnO_4 . Two experiments on tip regions gave the following results: 0.0176 and 0.0107 gm. oxalic acid per gram of leaf material.

RESULTS

The cells in *Elodea* leaves are arranged in longitudinal rows which may be readily traced along the length of the leaf. In five series of experiments counts were made of the number of crystals formed after stimulation in the tip regions of leaves at different positions on the stalk. Only the upper surface of the leaf was studied, and rows of cells ap-

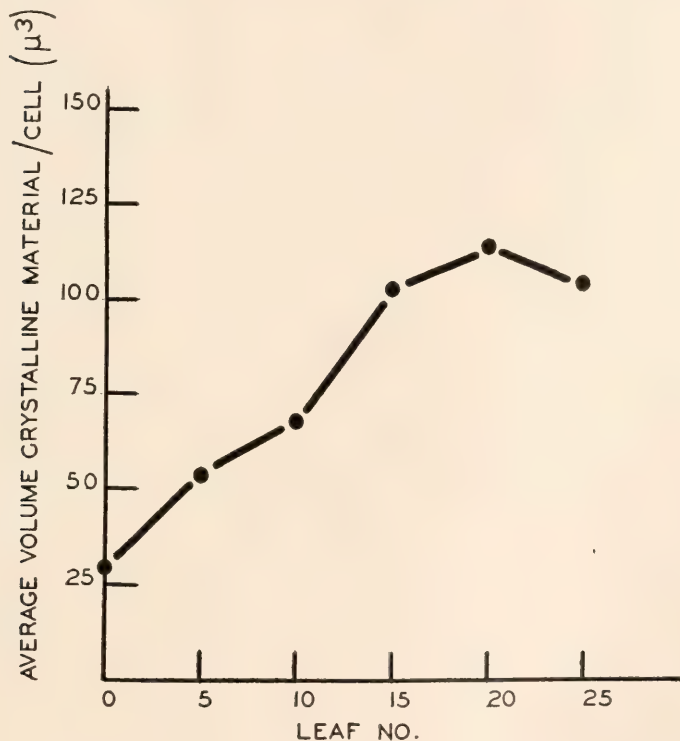


FIG. 1. Graph showing the average volume of crystalline material per cell formed after stimulation of *Elodea* leaves of different ages. The tip leaves are represented by 0, basal leaves by 25.

proximately halfway between the midrib and the edge of the leaf were selected for analysis. Beginning at the apex of the stalk every fifth leaf was removed and stimulated. Only the first 20 cells from the apex of the leaf were used and every fourth cell was counted so that for each leaf there were five cells counted. The computed data for the total amount of crystalline material in these experiments are shown graphically in Figure 1. Using the volume data mentioned previously it was determined that the various leaves contained the following amounts of crystalline material:

Apical leaf.....	29.5 cubic micra
Fifth leaf.....	55.7 " "
Tenth leaf.....	67.8 " "
Fifteenth leaf.....	102.3 " "
Twentieth leaf.....	114.0 " "
Twenty-fifth leaf.....	104.3 " "

In contrast with the group of apical leaves studied in which no tetragonal crystals were found, one large tetragonal crystal was noted in the fifth leaf group, one small tetragonal crystal in the tenth and fifteenth leaf groups, one large and five small tetragonals in the twentieth leaf group, and seven small tetragonal crystals in the twenty-fifth leaf group.

DISCUSSION

Mazia and Clark (1936) state that stimulation of *Elodea* cells causes a release of calcium from the cortex into the cell interior where the free calcium combines with the soluble oxalates in the central cell vacuole to form the crystals of calcium oxalate.

The experiments reported by the writer show clearly that there is an increase with age in the amount of calcium oxalate formed after stimulation of the cells of *Elodea*, and it may be concluded that the cell cortex of old *Elodea* cells contains more calcium than young *Elodea* cells. The quantitative analyses of the oxalate content of young leaves conducted by the writer (see section on methods) show that oxalates are present as at least 1 per cent of the total wet weight of leaf material. As this concentration of oxalate is far in excess of the concentration of calcium in living tissues, an increase with age in the amount of oxalates would have no effect on the amount of calcium oxalate formed after stimulation.

The question then arises whether there is actually an increase with age in the amount of calcium per unit of cell surface or whether there is an increase in cell surface which would account for the additional calcium in old cells. The cell measurements referred to earlier show a

7 per cent difference in cell surface between apical cells from tip and basal leaves. Obviously, a 7 per cent increase in cell surface is not adequate to account for the 300 per cent increase in the amount of crystalline material observed in the present experiments, and it may be concluded that the concentration of calcium per unit of cell surface increases with age in the cortex of *Elodea* leaf cells.

The results obtained in these experiments demonstrate that calcium increases with age in plants as well as mammals. The amount of cortical calcium per unit of plant cell surface increases with age.

SUMMARY

By means of electrical stimulation of the leaves of *Elodea canadensis*, insoluble crystals of calcium oxalate were formed in the central vacuole of cells of different ages. There was an increase with age in the amount of crystalline material formed; moreover, the amount of cortical calcium per unit of cell surface increased with age.

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INCREASE OF CORTICAL CALCIUM WITH AGE IN THE
CELLS OF A ROTIFER, *EUCHLANIS DILATATA*, A
PLANARIAN, *PHAGOCATA* SP., AND A TOAD,
BUFO FOWLERI, AS SHOWN BY THE
MICROINCINERATION TECHNIQUE¹

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In the preceding study the writer (1942) demonstrated that the amount of calcium in the cortex of the leaf cells of *Elodea* increases with age. The following studies were conducted in order to discover whether a similar increase in calcium content occurs in representative animal cells.

MATERIAL AND METHODS

Material for analysis was chosen so as to include both multiplying and non-multiplying cells of diverse organisms. The rotifer, *Euchlanis dilatata*, and the gastrocnemius muscle of the toad, *Bufo fowleri*, were selected to provide examples of non-multiplying cells; the planarian, *Phagocata* sp., provides within the same animal both multiplying and non-multiplying cells.

Euchlanis dilatata

The stock of this rotifer was developed from a single animal found in a pond near Bloomington, Indiana. The rotifers were cultured in the laboratory on pyrex depression slides containing two drops of culture fluid (Sonneborn, 1936). A number of newly-hatched rotifers were isolated and 40 of these animals were removed and fixed for histochemical analysis. The remaining animals were cultured on the depression slides throughout the entire life span. The animals were transferred daily to fresh depressions and samples of 20 animals were taken each day for chemical analysis. The last sample was taken at the end of the fourth day at which time the rotifers manifested the characteristic

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changes of senescence. The maximum age of *Euchlanis dilatata* when grown at 30° C. is four to five days. This was determined in a number of experiments on the normal life cycle (unpublished data).

Phagocata sp.

Several hundred of the multipharyngeal planarian, *Phagocata sp.*, were collected from a spring near Bloomington, Indiana, and brought into the laboratory. The planaria were examined with a dissecting microscope and measured while fully extended. The smallest animals found were 3 to 4 mm. in length; 25 of these small planaria were selected for chemical analysis. A second group of 25 animals 5 mm. in length and markedly broader than the preceding group of animals, was selected for analysis to represent the planaria of medium size, since these were most abundantly found. The largest animals were 10 mm. in length; 25 of these were selected at random for chemical analysis. On the basis of size it was assumed that the planarians 3 to 4 mm. in length were the youngest of the three groups and that the planarians measuring 10 mm. in length were the oldest. This conclusion was substantiated by a comparison of the degree of pigmentation of the animals. The smallest animals possessed the light gray pigmentation of young planaria while the large animals were a dense black, a characteristic of adult *Phagocata*.

Bufo fowleri

A collection of toads was made during the third week of August in the vicinity of Greenwood Lake, Indiana. All the animals were gathered from a relatively small area in order to reduce environmental variations to a minimum.

Six toads measuring 5.6 to 6.9 cm. in length (fully extended) were selected for analysis and pithed. The gastrocnemius muscles were carefully excised and were immediately put into absolute alcohol-formalin fixative. Six toads measuring 13.6 to 15.7 cm. were pithed and the gastrocnemius muscles excised and fixed as were the previous group. On the basis of the length measurements described above and the growth data of Hamilton (1934) whose work was done on the closely related toad, *Bufo americanus americanus* Holbrook, it was concluded that the toads measuring 5.6 to 6.9 cm. were four to five months old and that the toads measuring 13.6 to 15.7 cm. were two or more years old.

Microincineration Technique

The technique of microincineration employed in these experiments is essentially the same as that of Scott (1933a). Satisfactory results were

obtained by the author (1938) with this technique in an analysis of the localization of calcium in *Paramecium caudatum*. The experimental material was fixed in absolute alcohol-formalin (9 pts. to 1 pt.), dehydrated, cleared in xylol, and imbedded in paraffin. The rotifers and planaria were sectioned at six micra and the gastrocnemius muscle of the toad was sectioned at eight micra. The sections were mounted on clean glass slides and placed in an electric furnace. As recommended by Scott, the initial temperature increase was effected slowly, 20 minutes being required to obtain a temperature of 100° C. The temperature was then raised to and kept at 600° C. for 30 minutes which was found to be adequate for complete volatilization of the organic substances. The slides were cooled very slowly and placed without cover glasses in clean slide boxes to make possible future chemical analysis of the incinerated material. No attempt was made to analyze other elements besides calcium although some measure of the iron content of the preparations could be obtained from inspection of the untreated slides. A reddish-yellow color in the untreated incinerated preparations indicated the presence of iron and the intensity of this color could be used as an index of the iron content.

Calcium was identified chiefly by means of the alizarin reaction. (Saturated aqueous sodium alizarin sulfonate reacts with calcium to form the red precipitate of calcium alizarinate.) Occasional checks on the alizarin reactions were made by means of the delicate gypsum reaction. The reagents used in this reaction were one microdrop of 0.1 N HCl followed by one microdrop of 0.1 N H₂SO₄ (Scott). The presence of calcium was indicated by the formation of needle-like crystals of calcium sulfate. Since there was a marked diffusion of salts following the HCl treatment, precise localization of calcium could not be effected by this reaction, and for that purpose the alizarin reaction was depended upon completely. There was no piling up of the granules of ash in the incinerated sections since the material was cut at six and eight micra; that is, the incinerated preparations were but one granule deep. Consequently, an increase in the number of granules which stain red after the alizarin treatment, and thus an increase in the intensity of the staining reaction could be taken to mean that the amount of calcium had increased. Similarly, an increase in the number of calcium sulfate crystals formed, using the alternative test, was taken as an index of an increase in the calcium content.

Slides of the various organisms were stained with Ehrlich's haematoxylin, and these preparations were compared with the incinerated material for the purpose of identifying structures. As will be brought

out later in the paper, material stained with Ehrlich's haematoxylin may be used in direct determinations of the calcium content of the tissues with the intensity of staining as an index of the calcium content.

RESULTS

Bufo fowleri

The gastrocnemius muscle of the toad when incinerated furnishes a picture very similar to that obtained by Scott (1932) in his microincineration studies on striated muscle. The sarcolemma shows in young fibers as a very fine, white line of ash. The residual ash of the sarcolemma in many preparations is so slight as to be almost invisible. The ash of the sarcolemma in old muscle fibers is strikingly different from that of young tissue. Here the line of white ash is glaring and heavy. The striations show in young and old muscle fibers as parallel rows of discrete, small white granules. However, the granules in the striations of old muscle fibers are markedly larger than those in young muscle fibers. As observed by Scott, the isotropic bands or the J bands appear to be ash free. In these preparations no trace was found of the intermediate bands of the J striation, or the Z band, although Scott reports observing the ash skeleton of the Z striation in "favorable" material.

Tests with sodium alizarin sulfonate demonstrate that the nuclei of young and old muscle fibers are both rich in calcium. The large amounts of this cation present in the nuclei rendered it impossible to estimate quantitative differences between young and old muscle fibers. The sarcolemma of young muscle fibers after alizarin treatment shows the red color characteristic of calcium alizarinate and tests with the gypsum reaction reveal the needle-like crystals of calcium sulfate. Contrary to previous experience with other tissues it was possible to use the gypsum reaction for quantitative estimations of calcium because of the large size of the muscle fibers. The sarcolemma of old muscle fibers gives the same positive tests for calcium but consistently demonstrates a marked increase in calcium content, as indicated by an increase in the staining intensity with alizarin and an increase in the number of crystals formed in the gypsum reaction. Similarly, the striations of old muscles show a marked increase in the concentration of calcium over the striations of young muscle fibers. The nuclei of both young and old muscle fibers contain varying amounts of iron as indicated by the presence of reddish-yellow granules. Iron is also present in the striations of both young and old fibers and there appears to be an increase in the iron content of the striations with age.



Phagocata sp.

Incinerated preparations of this multipharyngeal planarian show an interesting variation in the total ash content of the various tissues. The epithelial lining of the body is richest in inorganics. The pharynges show a markedly low total inorganic content in contrast to the high salt content of the epithelium (Fig. 1). The ash in the pharynges is a dull bluish-white and is uniformly scant in the various tissue layers of these organs. However, the cell membranes of the cells lining the lumina of the pharynges contain considerably more ash than other regions of these organs. Throughout all the tissues and organs that were examined

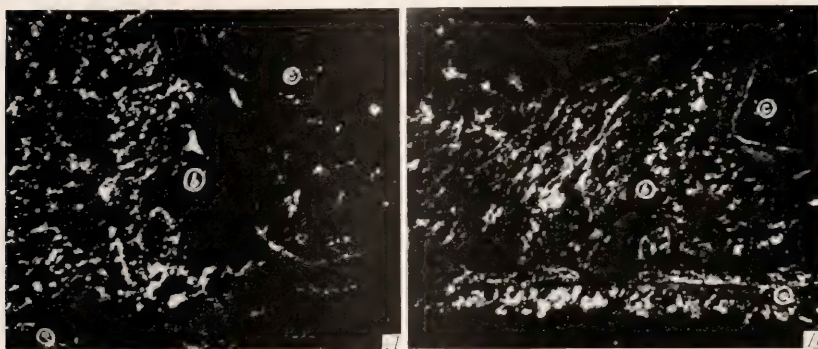


FIG. 1. Photomicrographs of incinerated preparations of *Phagocata*. Sections cut at 6 micra. (A) Young animal showing relatively large amounts of ash in the interstitial cells and small amounts of ash in the pharynges. (B) Old animal showing the increase in the ash content of the interstitial cells and the epithelial cells. Ash outline of some of the cells of the pharynges can be distinguished. Epithelium (a), Interstitial cells (b), Pharynx (c). Eastman "Contrast Process Panchromatic" film; 500-watt projection lamp; 15-second exposure, 200 magnification.

there was an increase in the total amount of ash with age. The basal membrane of the epithelial lining of the body in young animals is very thin and difficult to detect, but in old specimens the basal membrane is a glaring white line of ash. The increase in total ash content of the cell membranes of epithelial, interstitial (Fig. 1), and nerve cells is apparent. There appear to be no detectable changes in the total ash content of the rhabdites or the nuclei in the epithelial layer with age. This may be due to the fact that even young preparations are very rich in inorganics in these structures and an increase would be difficult to detect. The tissue layers of the pharynges, which as previously stated contain relatively little ash, show little or no increase with age in total ash content (Fig. 1).

Application of the chemical tests for calcium demonstrates that calcium increases with age in all the cells studied. The epithelial layer

is rich in calcium in young as well as old specimens, but there is a marked increase in the calcium content of the cell membranes of the epithelial cells and basal membrane with age. There is an increase in the calcium content of the nuclei and cell membranes of the cells of the pharynges with age, but the change here is far less than it is in the epithelial layer. Repeated tests for calcium in the cells of the pharynges confirm this slight change with age in the calcium content. The cilia of the lining of the pharynges are apparent in most of the preparations but no changes in their calcium content could be observed. The most marked increase in calcium content with age in the cells of the pharynges occurs in the cell membranes of the cells lining the lumina. A pronounced increase in the calcium content of the interstitial and nerve cells was observed. Calcium increases with age in the nuclei, cytoplasm, and particularly the cell membranes of these cells.

Examination of the untreated incinerated slides indicates that the epithelial and interstitial cells, and the rhabdites are richest in iron content. The epithelial and nerve cells show no increase in iron content with age. The interstitial cells reveal an inconsistent fluctuation in iron content with age. The cytoplasm of these cells, in some preparations, shows an increase in iron content with age while in other preparations there was no perceptible difference in the iron content of young and old specimens.

The picture obtained in the animals of medium size was very similar to that found in the group of large planaria. However, the concentration of calcium in the various cells of the large planaria was somewhat greater than that found in the cells of the medium group.

Euchlanis dilatata

Incinerated preparations of this rotifer manifest the same age changes that were observed in *Phagocata*. There was no significant difference in the amount of total ash in the cells of the one- and two-day-old specimens. However, the three-day-old rotifers contained much more ash than did the younger material, and the amount of total ash increased still further in the four-day-old material.

Tests with sodium alizarin sulfonate and the gypsum reaction showed that the increase in the calcium content of the rotifers paralleled the total ash increase. Thus, the first indication of an increase in calcium content was observed in the three-day-old material and a further increase was found in the four-day-old rotifers. Difficulty was experienced in identifying the skeletons of the various cells in this rotifer and only the cells of the brain, gut, and ovary showed clearly in the incinerated prep-

arations. This may be due to the fact that the animals contract somewhat during the fixation process. Tests indicated that calcium increased markedly in the nuclei, cytoplasm and cell membranes of these cells in the three- and four-day-old rotifers. The cell membranes of the one- and two-day-old rotifers showed as thin rings delicately tinted with a red color after they were stained with alizarin. The cell membranes of the three- and four-day-old rotifer material showed as heavy rings of ash which were stained a deep red with the alizarin.

The iron content of the cells of *Euchlanis* appeared to vary somewhat with age, but the iron changes were not as consistent as in *Phagocata*. There was a distinct increase in the iron content of some of the four-day-old specimens over the younger material. On the other hand, specimens were found in which the iron content was higher in the two- and three-day-old material than it was in the four-day-old material. The iron was found to vary most in the nuclei and cell membranes.

Ehrlich's Haematoxylin

It was earlier stated that control slides were prepared which were stained with Ehrlich's haematoxylin. Routine checks made with these slides on incinerated preparations revealed a striking relationship between the intensity of haematoxylin staining of the various cell types and the calcium distribution and content of these cells. In agreement with Scott (1933) it was found that structures rich in calcium as determined by microincineration invariably stained a dark purple with the haematoxylin, and structures with a low calcium content stained but lightly. Thus, the cells of the pharynges of *Phagocata* were delicately stained with haematoxylin in contrast to the other body tissues of *Phagocata* which stained heavily. As observed previously, the pharynges were very low in calcium content. The basal membrane of the epithelial layer of *Phagocata*, which analysis for calcium following microincineration proved to be rich in calcium, stained a dense purple with the haematoxylin. Similarly, the cell membranes of the epithelial and interstitial cells which were demonstrated to be rich in calcium also stained very heavily with the haematoxylin. A comparison of stained slides of young and old planarian and toad material revealed that the old material under the same staining conditions invariably stained more heavily than did the young material, and that the regions that stained more heavily corresponded to those regions which manifest an increase in calcium content with age. This relation between the intensity of staining with Ehrlich's haematoxylin and the calcium content has been frequently observed by the author in various types of material.

The use of haematoxylin as an indicator for calcium has been criticized by Lison (1936) in his excellent book on histochemical methods. He states that the color reaction between haematoxylin and calcium is dependent upon the presence of aluminum, chromium or gold in the fixatives used. However, despite the fact that the reaction is indirect, the close correlation between the calcium content of a structure and the intensity of haematoxylin staining of that structure justifies the use of this simple staining procedure for the identification and localization of calcium in cells and tissues.

CONCLUSIONS

The present studies demonstrate that a calcium increase with age, observed in the cortex of *Elodea* cells (Lansing, 1942), occurs similarly in the cells of two invertebrates and in the muscle tissue of a vertebrate. The widespread occurrence of this localized calcium increase with age suggests that it is a general characteristic of the aging process.

Further development of the relation between calcium and aging from both a descriptive and experimental point of view will require an objective quantitative method for the determination and localization of calcium in cells. Further, since it has been demonstrated that calcium increases with age in many tissues and organs of mammals (Lansing, 1942), it would be pertinent to determine whether the calcium increase with age in mammals also takes place in the cell membranes.

SUMMARY

A rotifer, *Euchlanis dilatata*, a planarian, *Phagocata* sp., and toad, *Bufo fowleri*, were selected for an analysis of calcium localization and of possible calcium increase with age. Samples of animals of different ages were sectioned, incinerated and examined with a darkfield microscope. Calcium was identified by means of sodium alizarin sulfonate and the gypsum reaction. It was demonstrated that calcium increased with age in the cell membranes of all the cells studied. Calcium also increased with age in the nuclei and cytoplasm of nerve and interstitial cells of *Phagocata* and the cells of *Euchlanis*. Because of the large amounts of calcium present in the nuclei it was difficult to determine the degree of calcium increase.

The iron content of the various cells appeared to vary considerably but inconsistently with age.

Control slides stained with Ehrlich's haematoxylin showed a correlation between the intensity of staining of structures and the calcium content of those structures. The suggestion was made that Ehrlich's haematoxylin may be used as an indicator for bound calcium in cells.

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THE HYPOPHYSIS OF THE BROAD-BILLED SWORDFISH, *XIPHIAS GLADIUS* L.

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The life history of the broad-billed swordfish (*Xiphias gladius* L.) is unknown, despite the investigations of marine biologists for a considerable period (Goode, 1873; Fish, 1926). Information concerning the habits of the adult fish, the location of its breeding grounds, or the occurrence of sexually mature swordfish is completely lacking. Therefore, a knowledge of the reproductive condition of the swordfish in all parts of its extensive range would be helpful in locating its breeding areas. The purpose of this paper is to determine primarily the aspect of the hypophysis of those swordfish which occur in southern New England waters. Their state of ovarian development has been described (Lee, 1942). It is hoped that these studies which have established the reproductive condition of those swordfish which are taken in this region, will be extended to include fish from all parts of its range, eventually locating its breeding grounds by this means.

The study of the hypophysis of the swordfish captured off the southern New England coastline was begun in the summer of 1940. Twenty-three pituitaries were obtained during this season, but the sex of the fish from which they were taken was undetermined. Therefore, the work was repeated in the summer of 1941 on glands from 13 swordfish, all of which proved to be female. Their pituitary glands were identical with those obtained during 1940.

METHODS

Dawson's and Friedgood's (1938) method of preparing the pituitaries of the rabbit and cat for study was used throughout this examination of the swordfish gland. This method was used successfully by Scruggs (1939) in his studies of the hypophyses of 16 different fish. The glands were fixed for 48 hours in ten parts of 0.1 per cent NaCl solution supersaturated with HgCl_2 , to which one part of formalin was added just prior to use. Serial sections were cut at three to four micra.¹

* Contribution No. 317.

Owing to the conditions of obtaining the pituitaries, an interval of from four to six hours often occurred between the capture of the fish and the fixation of the gland. However, all classes of cells appeared maintained, cytoplasmic granules were preserved, the vacuolar membranes were intact and negative images of the Golgi apparatus were readily found. The fixation compared favorably with that obtained for other fish pituitaries fixed immediately by the same method (Scruggs, 1939) and with one swordfish pituitary fixed less than 40 minutes after capture of the fish. The relatively good preservation of the material was probably due to the fact that the heads were removed and packed in ice below decks upon capture of the swordfish.

RESULTS

The hypophysis lies beneath the infundibular region of the brain in a deep sella turcica, which is lined with periosteum and with that portion of the meninx that descends to form a tightly fitting sheath about the gland. As in higher organisms, the dorsal surface of the gland is covered by a diaphragm of the meninx, which is reflected to form a collar of connective tissue between the hypothalamus and the pituitary. There is no saccus vasculosus, nor any structure which may be likened to it, in the swordfish.

The pituitary gland is relatively large with respect to the brain. In four specimens chosen at random, the gland averaged 0.13 cc. in volume and weighed 0.08 gm., wet weight. In shape it resembles a blunt tetrahedron with its base pressed closely against the infundibulum. One surface faces anteriorly and the other two extend postero-medially to form the posterior margin of the gland. From the corners and apex of the hypophysis, extensions of the meninx adhere to or fuse with the periosteum, binding the gland tightly into the sella.

Sagittal and frontal sections of the pituitary reveal that the gland is divided into the four usual regions described by Stendell (1914) and other investigators of the teleost pituitary gland: the pars nervosa, the pars intermedia, the pars anterior, and the Übergangsteil; but the topographical distribution of these regions varies from the customary arrangement.

¹ The brain of the broad-billed swordfish is exceptionally small in relation to the size of the body. The average brain weight was 1.9 gm. and the average length was 3.5 cm. in four specimens chosen at random, whose average weight was 160 gm. and whose average total length was 2.8 m.

The pars nervosa

There is no discrete stalk of nervous tissue connecting the hypophysis to the brain. A slender tongue of the infundibular recess descends through the ventral medial sulcus, then expands laterally to form a small secondary cavity of an inverted mushroom shape. From the thin ventral wall of this cavity, which lies directly in contact with the dorsal surface of the hypophysis, the pars nervosa penetrates the pars glandularis. The pars nervosa appears primarily as a thick stalk of nervous tissue extending into the center of the hypophysis. Here it arborizes completely into a tree-like structure which is entirely surrounded by the pars intermedia. Accessory penetrations of tissue from the infundibular region project into the gland, peripherally to the main nervous stalk. The exact nature of these secondary penetrations is unknown, but they appear to be combinations of connective tissue and blood vessels from the meningeal diaphragm, with slender trabeculae of nervous tissue which descend directly from the infundibulum. The pars nervosa possesses the fibrous nature reported by Bock (1928), Charipper (1936), and others for this region of the teleost pituitary gland. The nuclei of connective tissue cells are common; but the chromatic granules, or Herring bodies, such as were found by Scruggs (1942) in the pars nervosa of the goldfish and carp, were not found in this portion of the swordfish pituitary.

The cells in the pars anterior of the mammalian pituitary are described as "acidophiles," "basophiles," or "chromophobes," depending upon whether they assume the so-called "acid" stains such as eosin, orange G, or azocarmine, the so-called "basic" stains such as methyl blue, or assume no stain whatever. This terminology has been transferred to cells within the three subdivisions of the teleost pituitary gland, which react in apparently similar fashion, by Matthews (1936), Scruggs (1939), Kerr (1940) and other investigators. A like procedure has been adopted in the description of the component cells of the swordfish hypophysis. Those pars intermedia cells which stain with orange G are referred to as "pars intermedia acidophiles," etc.

The pars intermedia

The pars intermedia appears as a cone-like region with its apex directed ventrally which completely surrounds the arborized portion of the main nervous penetration. This region of the hypophysis is characterized by predominant weak basophiles which assume a dull blue color. These cells are small, being six to eight micra in diameter, and

are generally iso-diametric. Occasionally basophiles are found in the pars intermedia which are twice the usual size and possess small carminophilic granules distributed throughout the cytoplasm. These granules may be mitochondria, for those in proximity to the nucleus often assume rod-like forms. In addition to the basophiles, orange G acidophiles are also found in the pars intermedia, but these cells are much fewer in number. The cytoplasm of the acidophiles is of a dull orange color, with a fine granulation. Negative images of the Golgi apparatus are often seen in this type of cell. There is no definite arrangement of the acidophiles within the pars intermedia of the swordfish, as Scruggs (1939) and Bell (1937) found in the goldfish hypophysis, for they seem scattered at random throughout this part of the gland.

Degranulated cells, with an indistinct outline and a partially pycnotic nucleus, occur throughout the pars intermedia. These strongly resemble similar cells described by Scruggs (1939) for the pars intermedia of the majority of teleost pituitaries that he studied.

The Übergangsteil, or "transitional region"

The main portion of this part of the hypophysis is wedge-like in sagittal section, and lies dorsally to the base of the cone-shaped pars intermedia, with the main mass of tissue in the postero-dorsal corner of

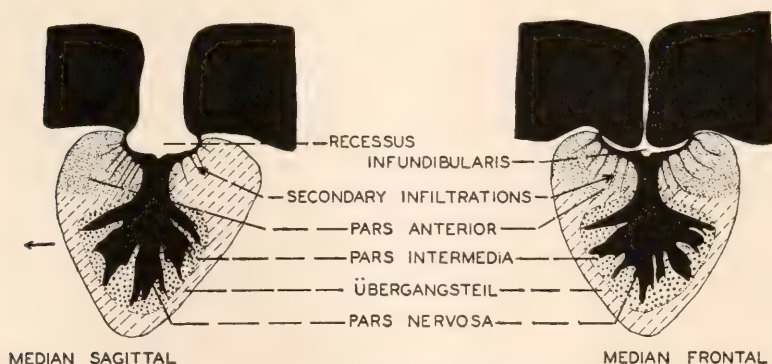


FIG. 1. Diagrams of median sagittal and median frontal sections of the swordfish pituitary gland, showing the topographical arrangement of the glandular regions.

the gland (Fig. 1). From this region paired processes extend anteriorly around the main nervous penetration to the frontal surface of the pituitary. A sheath of Übergangsteil material also descends over the pars intermedia, completely enveloping it and forming the superficial layer

of the hypophysis, excepting that portion of the gland where the pars anterior is situated.

The cells of the Übergangsteil are of two types; one is an acidophile and the other a basophile. The acidophiles are strong carminophiles, which react readily with and retain the azocarmine to assume a deep crimson color. Their cytoplasm is finely granulated, with larger granules dispersed through the cell. Usually the acidophiles are clustered about the capillaries, which are most numerous in this region of the gland; they also border the penetrations of infundibular material. The basophiles of the Übergangsteil, in those fish captured in midsummer, are of a brilliant blue color, with homogeneous cytoplasm and an exceptionally large cytoplasmic vacuole in each cell. (See plate.) The entire Übergangsteil appears net-like in sagittal section, due to the presence of these vacuolated basophiles.

The pars anterior

The pars anterior occupies a horse-shoe shaped region in the antero-dorsal portion of the hypophysis, with its arms extending posteriorly around the zone of contact of the infundibulum with the pars glandularis to meet the main Übergangsteil mass. There are two types of cells, an amphiphile and an acidophile, in the pars anterior of the swordfish. The amphiphiles react polychromatically with the triple stain, showing a predominant tendency to assume the anilin blue which gives them a bluish-purple color. These cells line the infiltrations of infundibular material which penetrate this part of the gland. Their nuclei adopt a distal position with regard to the infundibular tissue; the intervening cytoplasm is very finely granulated and usually possesses small vacuoles.

The bulk of the pars anterior is composed of dark red carmine acidophiles. These are readily distinguished from the type of acidophiles in the Übergangsteil by means of their predominant color and coarsely granulated cytoplasm. In those acidophiles which approximate the numerous capillaries in the pars anterior, there is a definite concentration of these granules in the ends of the cells proximal to the blood vessels, with the nuclei occupying a distal position.

Histological Changes in the Pars Glandularis

During both summers that the swordfish pituitaries were studied, considerable changes were found in the aspect of the pars intermedia and the Übergangsteil, as the season progressed.

The pars intermedia of pituitaries obtained in early July possessed one to two orange G acidophiles per high power microscopic field ($490\times$). The numbers of these cells gradually increased in pituitaries obtained successively throughout the summer, until there were 10 to 11 such acidophiles present per high power field in the pars intermedia of the last fish captured in early September. As the acidophiles increased in number, they appeared to be most numerous in the regions of the pars intermedia closest to the main branches of the pars nervosa which it surrounds. In this respect, the pars intermedia and its components in the swordfish agree more closely with the findings of Bell (1937) and Scruggs (1939) mentioned previously.

The basophiles of the Übergangsteil also showed marked changes in appearance during both summers. The aspect of these cells in pituitaries taken from late July to the end of the season has been described previously. However, there is a relatively complete absence of vacuoles in the Übergangsteil basophiles of pituitaries obtained in early July, and the cytoplasm of these cells possesses a coarse granulation. (See Plate.) As the sex of those swordfish from which these pituitaries were taken was undetermined, the exact nature and causes of the differences in aspect of the Übergangsteil basophiles still remain to be investigated.

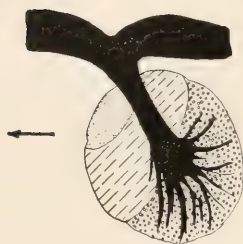
DISCUSSION

The topographical appearance of the swordfish hypophysis presents an interesting comparison with that of teleost pituitary glands that have been studied previously (Stendell, 1914; Bock, 1928; Charipper, 1936; Bell, 1937; Scruggs, 1939) with regard to the Übergangsteil. The generalized teleost pituitary is composed of two primary regions: that of nervous origin, the pars nervosa, and that of buccal origin, the pars glandularis. In the latter, the Übergangsteil develops as a plate or disc-like collar around the more dorsal portion of the pars nervosa, situated more or less completely between the pars intermedia and the pars anterior. However, in the swordfish the Übergangsteil is considerably flattened in its frontal portion, with the bulk of its tissue in the postero-dorsal region of the gland. A sheath of its components also invests completely the pars intermedia, to form the superficial layer of the hypophysis (Fig. 2). This condition suggests that in organogeny the process of intergrowth and differentiation of the pars glandularis with the pars nervosa is incomplete, as compared to the same process in other teleosts. Scruggs (1939) found a topographical condition similar to that of the swordfish hypophysis in the *Centrarchidae*; but in these fish the Über-

gangsteil sheath is fragmentary or completely lacking in the postero-medial line.

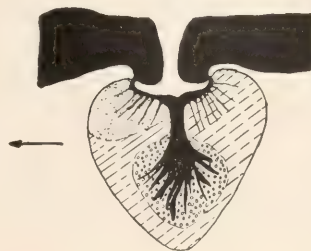
Because of differences in methodology, it is extremely difficult to compare the cellular components of the swordfish hypophysis with those of other teleosts that have been studied. A comparison of the nature of the subdivisions in the pars glandularis is probably valid, however, if made with regard to the predominant cell types present (basophiles, chromophobes, etc.), and to their distribution within the region in which they occur. Scruggs (1939) used the azan technique extensively on teleost pituitaries; hence the cellular components of the swordfish pitui-

GENERALIZED HYPOPHYSIS OF TELEOST



P. NERVOSA ——— ■
P. INTERMEDIA ——— ▨

SWORDFISH HYPOPHYSIS



P. ANTERIOR ——— □
ÜBERGANGSTEIL ——— ▨

FIG. 2. Diagrams comparing the topographical arrangement of the glandular regions in the swordfish pituitary gland with that in the generalized teleost pituitary. (Anterior end to the left.)

tary gland may be compared more closely with those of the teleost glands which he studied.

The cell types of the pars intermedia compare favorably with those described by Charipper (1936), Bell (1937) and Scruggs (1939). Weak basophiles characterize the region, with weak acidophiles scattered through them, more or less at random. Occasional chromophobes are evidently formed by degranulation of the basophilic cells. The fact that Dawson (1940) found these three types of cells in the pars intermedia of *Protopterus aethiopicus* indicates that this region of the fish pituitary gland is relatively constant with regard to its cell components.

The components of the Übergangsteil also agree with the general condition found by other students of the teleost pituitary gland. Its brilliant carminophiles and deep blue basophiles are essentially the same

types of cells that Scruggs (1939) found in the Übergangsteil of all fish that he studied save the flounder and stickle-back. Charipper (1936), Kerr (1940) and others are agreed that this part of the teleost hypophysis is usually highly chromatic, possessing strong acidophiles and basophiles with the various staining techniques.

The predominantly chromatic nature of the pars anterior, however, is at variance with the condition that Scruggs (1939) describes for the large majority of fish that he examined. Bell (1937) has shown that the pars anterior of the goldfish is dominated by orange G acidophiles; while Kerr (1940) found this region of the trout pituitary to be chiefly acidophilic, possessing a brownish-red acidophile when stained with Mallory's triple stain. Thus the pars anterior of the teleost pituitary gland may vary considerably in different species and genera.

In its fibrous appearance the pars nervosa of the swordfish hypophysis resembles that of other fish that have been examined; however, the chromatic granules described by Scruggs (1939, 1942) for the pars nervosa of many species, and by Evans (1940) for the eel, are not found in the swordfish.

The changes in aspect of the Übergangsteil and pars intermedia of the swordfish hypophysis are without precedence in the literature. However, several investigators have reported various dissimilar phenomena in the pituitaries of other fish. Matthews (1939) and Scruggs (1939) have found several changes in the pituitary gland of *Fundulus heteroclitus* that are correlated with the beginning of the breeding season. Scruggs (1942) describes marked changes in the appearance of the pituitaries of *Cyprina carpio* and *Carassius auratus* that appear prior to and during the breeding periods of these fish. Evans (1940) reports large colloid-filled sinusoids in the pars intermedia of the eel, appearing

PLATE I

FIG. 3. The forward process of the Übergangsteil of a swordfish pituitary gland obtained on July 12, 1941. The darker cells are the bright crimson carmine acidophiles; the lighter cells are the granular basophiles. (720 X.)

FIG. 4. The forward process of the Übergangsteil of a swordfish pituitary gland obtained on August 4, 1941. The basophiles possess large cytoplasmic vacuoles. Their nuclei are slightly pycnotic, and their cytoplasm has lost its granulation, to assume a dense homogeneous nature. This condition prevails for the remainder of the season. (800 X.)

FIG. 5. The layer of amphiphiles in the pars anterior of a swordfish captured on July 24, 1940. The amphiphiles border an infiltration of connective tissue and blood vessels from the infundibular region. (1080 X.)

FIG. 6. Carmine acidophiles bordering a blood vessel in the pars anterior of a swordfish captured on July 24, 1940. (The same fish whose pars anterior is shown in Figure 5.) The large carminophilic granules are concentrated in that end of the cells nearest to the blood vessel. (1080 X.)

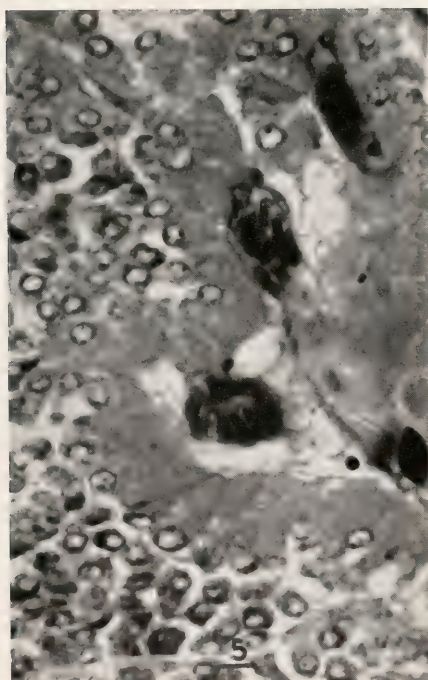
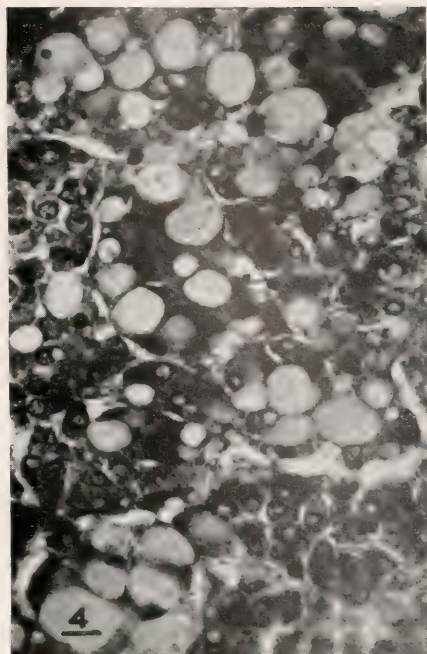
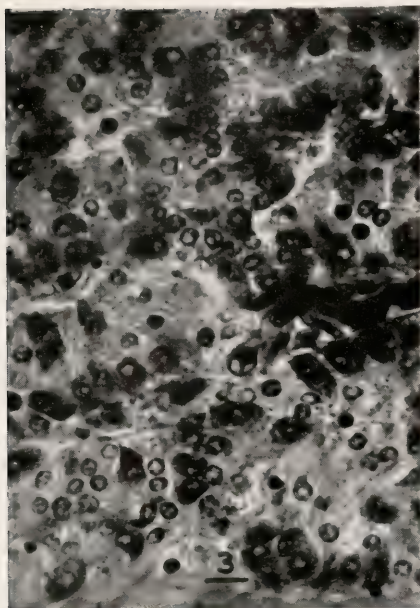


PLATE I

just before the onset of the migration impulse, with an enlargement of the pars anterior found at approximately the same time. Although these changes are not similar to those found in the pituitary of the swordfish, they indicate the components of the teleost pituitary gland may undergo a considerable modification which is apparently correlated with the breeding period. As the ovaries of the swordfish show no indication of maturation or of an increased pituitary activity during the brief time that the fish is found in southern New England waters, and as the exact nature of the changes found in its pituitary gland is still to be established, we can draw no conclusions regarding the time or the regions of breeding activity of the swordfish at present. Future examinations of the reproductive condition of this fish in all portions of its range will undoubtedly aid in the solution of this problem.

I am deeply in debt to Dr. A. B. Dawson of the Harvard Biological Laboratory for encouragement and aid in pursuing this study; to the staff members of the Woods Hole Oceanographic Institute, for their generosity in providing laboratory space and equipment; and to Mr. Samuel Cahoon of Woods Hole, whose facilities and knowledge of swordfishing were invaluable in securing the material.

SUMMARY

Twenty-three swordfish pituitaries were studied in 1940. It was impossible to determine the sex of these fish; therefore the work was repeated in 1941 on pituitaries from 13 swordfish, all of which proved to be female. Their pituitaries were identical with those obtained in 1940.

The swordfish hypophysis is a blunt pyramidal structure, situated with its base in close approximation with the infundibulum. The pars nervosa descends from the infundibular floor, as a main or primary infiltration which arborizes in the ventral portion of the gland, with smaller secondary infiltrations of infundibular tissue penetrating the more dorsal regions. It is fibrous in nature, with the nuclei of connective tissue cells occurring frequently. Herring's bodies are not found in the pars nervosa, and there is no evidence of migration of cells from other glandular regions into it.

The pars intermedia surrounds the arborized part of the main nervous penetration, as a cone-shaped region with its apex directed ventrally. There are three types of cells in this part of the gland; an orange G acidophile, a weak blue basophile, and a chromophobe. Carmine granules are found in many of the basophiles. The acidophiles

become more numerous in glands obtained successively during the summer.

The Übergangsteil is a wedge-shaped region, lying dorsal to the base of the cone-like pars intermedia, with its main portion in the postero-dorsal part of the hypophysis. A sheath of its material completely envelops the pars intermedia. The Übergangsteil possesses two types of cells: a strong carminophile with finely granulated cytoplasm, and a deep blue basophile with coarsely granulated cytoplasm. During the latter half of the summer the basophiles contain large vacuoles which first appear about the middle of July.

The pars anterior occupies a horse-shoe shaped region in the antero-dorsal part of the hypophysis. Its arms extend posteriorly toward the main mass of the Übergangsteil, often surrounding the zone of contact of the infundibulum and the pars glandularis in collar-like fashion. The pars anterior of the swordfish pituitary has two chief types of cells: an amphiphile and an acidophile. The former are of a bluish-purple color, and are found bordering the secondary infiltrations of infundibular material that enter this region. The acidophiles are coarsely granulated carminophiles, which lie beneath the more peripheral amphiphiles to make up the bulk of the pars anterior.

The 13 swordfish from which gonads were obtained in 1941 were females. The ovaries contained ova which did not change appreciably in diameter during the summer. Thus there is no evidence of sexual maturation of swordfish taken in southern New England waters.

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ACCUMULATION AND DISCHARGE OF SPAWN BY OYSTERS LIVING AT DIFFERENT DEPTHS

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(Fish and Wildlife Service, Fishery Biological Laboratory, Milford, Connecticut)

INTRODUCTION

While conducting studies on the biology of the oyster, *Ostrea virginica*, of Long Island Sound, an opportunity presented itself to observe and compare the condition of the gonads of these animals living at different depths. Such a study is of interest since the literature does not offer any comparative data pertaining to the accumulation of spawn and spawning activities of oysters existing in shallow and deep water. There are in the literature, however, several references which indicate that the oysters of shallow water discharge their spawn earlier than those of deep water. For example, Churchill (1920) states that the spawning period of *O. virginica* is regulated by the depth of the water and the general meteorological conditions. Nelson (1928), working in New Jersey, also expressed the opinion that oysters in shallow water spawn before those in greater depth. Neither of the two above-mentioned investigators, nor other workers making similar statements, offered any comparative data in support of their observations. Furthermore, no suggestion was offered as to what should be considered as shallow or deep water.

In Long Island Sound very shallow waters are not suitable for oyster cultivation because wave action during storms may shift and often destroy the oysters. On the other hand it becomes increasingly difficult to cultivate the beds and take care of the oysters in waters deeper than 30 or 35 feet. As a result of these conditions the greater part of the oyster grounds of Long Island Sound is confined to the depths ranging from 10 to 30 feet. Therefore, in these studies it was decided to confine observations to the same depth range.

METHODS

These studies were conducted in the summer of 1941. There were 14 sampling stations established in the oyster-growing area confined between Bridgeport and New Haven harbors. Of this number, four stations were located at a 10-foot depth, six stations at a 20-foot depth,

and four stations at a 30-foot depth. It is proposed to regard the above mentioned groups of stations as shallow, medium deep, and deep, respectively.

The collection of samples for observations on the average thickness of the gonadal layer of oysters living at the three different depths was begun on June 11 and continued until September 3. At each station a dredge-load of oysters was brought up and a sample of ten adult animals of four or five years of age was taken from it at random. Thus, 140 individuals were examined on each trip. The body of each oyster was cut transversely on the right side along a line extending through the stomach on a level with the lower edge of the palps, and the thickness of the gonadal layer was measured with a caliper. To determine the time of the beginning of spawning of the oyster population, numerous other samples were taken at very frequent intervals during the early part of July.

Several days after the beginning of spawning, collection of additional samples of oysters for determination of the condition of their gonads was initiated. Each sample now consisted of 20 individuals: thus during each trip 280 oysters were collected from 14 stations. The oysters were opened, dissected, and the condition of their gonads determined. On the basis of such an examination the oysters were classified as ripe-but-unspawned, less-than-half spawned, more-than-half spawned, and completely spawned.

OBSERVATIONS

The first measurements of the gonads of oysters made on June 11 showed that the gonadal layer was very thin and that the majority of the animals was unripe. Oysters of the shallow stations were the most advanced, the mean thickness of the gonadal layer of that group being slightly over 1.0 mm. (Fig. 1). Two other groups of oysters collected from the stations located at depths of 20 and 30 feet showed the thickness of the gonadal layer as about 0.9 and 0.8 mm, respectively. In all groups, especially in that of the deep water stations, there were individuals whose gonads only recently had changed from the wintering stage, and whose gonadal layer was extremely thin. Later in June the increase in the thickness of the gonadal layer of all three groups of oysters became quite rapid, and early in July, before the beginning of spawning, the gonads reached their maximum development.

Throughout the prespawning period the oysters of the more shallow stations possessed a heavier layer of spawn than the oysters from deeper water (Fig. 1). This difference became more apparent as the season

progressed toward the commencement of spawning. On July 2 the thickness of the gonadal layer of shallow water oysters was approximately twice that of oysters from the 30-foot depth. Oysters of medium deep stations occupied a position between the extremes.

Because the last measurement of gonads during the prespawning period was made on July 2, approximately one week before the beginning of spawning, it must be assumed that during that last week a further increase in thickness of the gonadal layer occurred. Therefore, it is probable that the maximum thickness of the gonadal layer of the oysters at all three depths was greater than is shown in Figure 1. However, judging by the examination of unspawned individuals collected on July

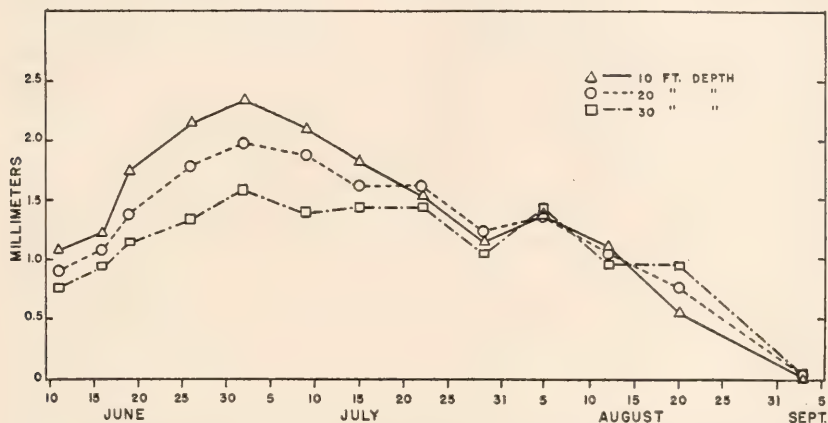


FIG. 1. Mean thickness of the gonadal layer of the oyster at three different depths.

9, the increase in thickness of the gonadal layer during the week elapsing between July 2 and 9 was very small.

In 1941, the beginning of spawning of the oyster population occurred about July 9. Spawning was of a general nature taking place at all the stations regardless of depth. After the beginning of spawning the thickness of the gonadal layers of oysters at all the depths began to decrease. While such a decrease was rather slow and gradual, it was most pronounced in the case of the shallow water oysters (Fig. 1). This, of course, indicated that the shallow water group discharged the spawn in larger quantities and more rapidly than the oysters of deeper water. As a result of the uneven rate of the discharge of spawn, the thickness of the gonadal layers of the oysters of all three depths became actually the same by July 22. Later on, the shallow water oysters began to possess less spawn than the two other groups (Fig. 1).

On July 15, several days after the beginning of spawning, a series of observations on the condition of gonads of the oysters was begun. It was found that the majority of the oysters at all the stations had already released part of their sex cells. It was of interest to note that, contrary to expectations, there were fewer ripe-but-unspawned oysters at the deep water stations than at more shallow ones (Fig. 2*A*). In all cases, nevertheless, the majority of oysters examined at this date were in the less-than-half spawned stage. Such animals constituted 71.0, 61.7 and 81.3 per cent of the samples collected at depths of 10, 20 and 30 feet respectively (Fig. 2*B*). More-than-half spawned oysters were most numerous at the 20-foot depth where they constituted 18.3 per cent of the total number of oysters examined (Fig. 3*C*). Completely spawned oysters were absent in the samples collected at medium-deep and deep stations, but at the shallow stations 1.6 per cent of oysters were identified as entirely spent (Fig. 3*D*). This observation indicated that among the oyster population living in shallow water there was a small group of individuals whose spawning season lasted but a single week. Several oysters of this type were prepared for histological examination which showed by the shrinkage of the gonadal follicles, phagocytosis, and the invasion of the cells of the vesicular connective tissue into the intra-follicular spaces that their gonads were in the typical post-spawning stage.

Within two weeks after the first examination, ripe-but-unspawned oysters showed a marked decrease in numbers. Such a decrease was noted at all three depths (Fig. 2*A*). Nevertheless, unspawned individuals were found in the samples collected at the 10-foot depth until August 12, and at the two greater depths until August 20. However, between July 29 and August 20, ripe-but-unspawned oysters never constituted more than 2.6 per cent of the population at any depth.

Early in August, approximately 50 per cent of the oyster population had half its spawn already discharged (Fig. 4). Again it was noted that the shallow water oysters, living at the 10-foot depth, reached this stage several days earlier than the animals living in deeper water (Fig. 2*B*).

As is shown in Figure 2*B*, the half-spawned stage was reached by the oyster population sometime between August 5 and 12. To determine a more exact date for the midpoint of the spawning period, the following method was employed: the oysters composing ripe-but-unspawned and less-than-half spawned classes were combined in one group, while two other classes, in a more advanced condition, were taken as the second group. By employing such a classification all oysters were divided into two groups of which the first contained all less-than-half spawned

oysters, while the second group was composed of the individuals which had more than half their spawn already discharged. The last group, of course, included all completely spawned oysters. When the percentage of these two groups was plotted against the time, two curves intersected at the point corresponding to August 8 (Fig. 4). Therefore, it can be assumed that this date represents the midpoint of the spawning season or, in other words, the date at which the oyster population of the Sound had discharged approximately one-half its spawn.

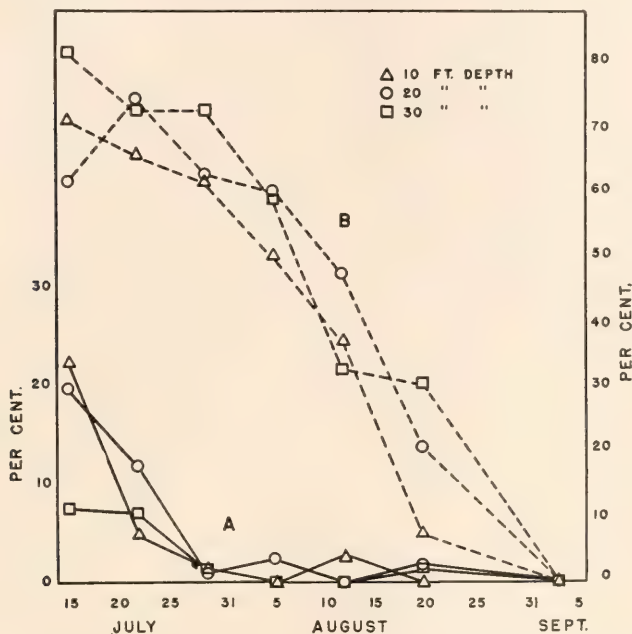


FIG. 2. Percentage of ripe-but-unspawned (A), and less-than-half spawned (B) oysters at each of three depths.

The conclusion that the midpoint of the spawning season lies close to the date of August 8 is further supported by the data obtained from the measurements of the thickness of the gonadal layer of oysters (Fig. 1). These measurements showed that the average thickness of the gonadal layer of the oysters recorded several days before the beginning of spawning was about 2.0 mm. On the other hand, the average thickness of this layer, as determined on August 12, the date nearest to August 8, was only about 1.0 mm., or approximately one-half that at the time of the maximum development.

Individual oysters containing more than half the spawn were still quite numerous in the samples long after the midpoint of the spawning period was reached (Fig. 2*B*). Even as late as September 20, 32.5 per cent of the oysters living at the depth of 30 feet was found in this stage. At more shallow stations their numbers were smaller.

As was stated before, few completely spawned oysters were found in the samples as early as July 15. Those animals, however, came from

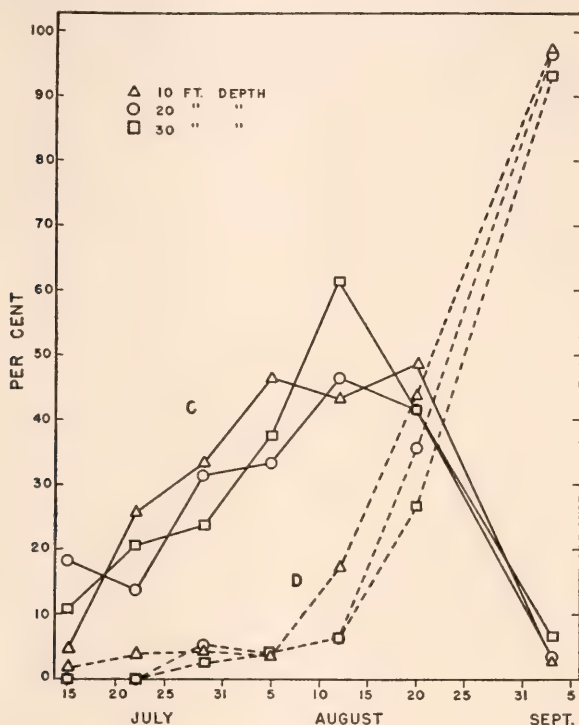


FIG. 3. Percentage of more-than-half spawned (C), and completely spawned (D) oysters at each of three depths.

shallow water. At deeper stations located at the 20- and 30-foot depths, completely spawned animals were first found on July 29 or two weeks later than at the shallow stations. At that date their numbers were comparatively small and never constituted more than 5.1 per cent of the entire population (Fig. 3*D*). A significant increase in the numbers of completely spawned oysters of shallow water was noted by August 12, when they constituted 17.1 per cent. At the two other depths, large numbers of spent oysters were observed one week later, when they constituted 35.8 and 26.6 per cent of the population living at 20- and 30-foot

depths respectively. After August 20, the number of completely spawned oysters rapidly increased and on September 3, at the end of the observation period, over 95.6 per cent of the entire population belonged to that group (Figs. 3 and 4). The remaining 4.4 per cent represented the animals still containing small quantities of spawn and, therefore, considered as more-than-half spawned but not entirely spent. It may be

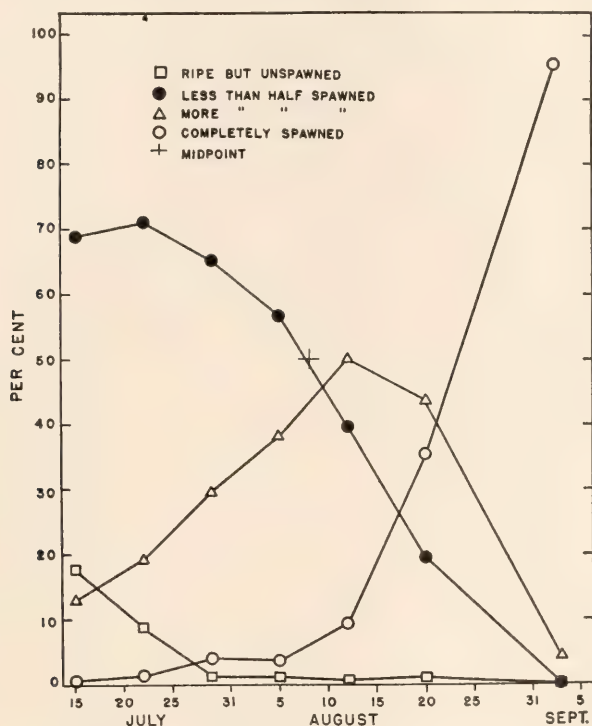


FIG. 4. Percentages of ripe-but-unspawned, less-than-half spawned, more-than-half spawned, and completely spawned individuals composing the oyster population of the depths ranging from 10 to 30 feet. July 15 to September 3, 1941. Cross sign (+) indicates the midpoint of the spawning season.

concluded, however, that the spawning activities of the majority of the oyster population living at the depths ranging from 10 to 30 feet were finished about September 3.

DISCUSSION

The observations conducted in 1941 in Long Island Sound indicated that, within the depth range of from 10 to 30 feet, the spawning of the oyster population began at the same time. This observation is in agree-

ment with those of previous years when it was found that the oysters of shallow and deep water beds approached ripe conditions and discharged spawn on or about the same date (Loosanoff, 1939; Loosanoff and Engle, 1940). The spawning season continued from about July 9 until about September 3. The shallow water oysters finished spawning somewhat earlier than the oysters living in deeper water.

It was determined that the midpoint of the season, or the date by which the oyster population had discharged approximately one-half the spawn accumulated, was reached by August 8. Again shallow water oysters reached this point several days earlier than the animals living in deeper water.

It is of interest to know that, among the oyster population studied, there was a small group found whose spawning activities were begun and ended within a week's time. It is possible that these individuals represent a race physiologically different from other local oysters whose spawning periods extend about two months. It has been suggested that such individuals are the descendants of the oysters brought on several occasions to Long Island Sound from northern waters where the spawning season is of very brief duration.

The presence of ripe-but-unspawned oysters, encountered in the samples collected six weeks after the beginning of the spawning season, presents an interesting problem. The failure of some individuals to spawn until very late in the season was first noticed during the spawning season of 1937 (Loosanoff and Engle, 1940). Physiological ripeness of such a late-spawning type of female oysters was established by fertilizing their eggs with spermatozoa taken from the males of the same late-spawning group. Therefore, it appears that under natural conditions some of the ripe oysters cannot be induced to spawn by a relatively high temperature and by the chemical stimulation caused by the presence in the water of the sex products discharged by the oysters living nearby. These field observations are supported by the laboratory experiments of Galtsoff (1940) who found that from 5.4 to 22.8 per cent of males and from 4.7 to 29.4 per cent of females could not be induced to spawn by the combination of thermal and chemical stimuli. Histological examination of these animals showed that a great majority of them was ripe.

The observation that the oysters living in shallow water develop much larger quantities of spawn than the oysters of greater depths is of considerable significance. It indicates that during the prespawning season it may be advantageous to have large numbers of oysters concentrated in relatively shallow water. In that case, theoretically, each oyster would produce about twice as many sex cells as it would if it were kept

at the depth of 30 feet. At present no satisfactory explanation can be offered as to why the shallow water oysters accumulate larger quantities of spawn. At first the difference in water temperature was suspected as causing much more favorable conditions of existence for shallow water individuals. Field records showed that during the prespawning period, the bottom water temperature at shallow stations was from only 1° to 2° C. higher than at the 30-foot depth. Therefore, even if the difference was actually insignificant, the assumption could be made that the shallow water oysters accumulated more spawn because they lived in warmer water. Such an assumption, however, would be contradictory to the observations of the last nine years which showed rather conclusively that the quantity of spawn developed by oysters in different years could not be correlated with the deviations of temperature during spring and early summer above or below normal (Loosanoff and Engle, 1940). Furthermore, minor differences in temperature have been found to be unimportant in other physiological activities of oysters. For example, Galtsoff (1928), in well controlled experiments performed on a large number of oysters, found that within temperature limits ranging from 13° to 22° C. there was no visible effect of temperature on the opening and closing of shells. Recent experiments on the feeding of oysters conducted by the authors at Milford Laboratory failed to show that, within the temperature range mentioned above, there was a well defined decrease or increase in the rate of feeding of oysters when the temperature of the water was changed by one or two degrees.

The differences in the salinity of the water at the stations of the three different depths were rather small seldom exceeding one part per mille. Evidently such a difference was not significant enough to cause much heavier development of the gonad material in the oysters living at one of the depths. It is thought, therefore, that some other conditions are responsible for the accumulation of greater quantities of spawn by shallow water oysters. The suggestion is offered that it may be due to the presence of larger numbers of food organisms in the shallow zones. Unfortunately the present knowledge of the feeding habits of oysters is rather limited. Even though there are many articles describing the plankton organisms found in the oyster's stomach, little is being said in these articles on the digestion and assimilation of such forms. As a result, very little information is available as to the quantities and qualities of various food organisms assimilated by oysters. Therefore, this phase of the biology of the oyster is not clearly understood. Obviously, in order to ascribe the greater gonad development of oysters of certain depths to their feeding habits and the presence of certain food organisms, further research along these lines is necessary. At present a number of

experiments and observations devised to provide the possible answer to such problems are being conducted at the Milford Laboratory.

SUMMARY

1. Prior to spawning the thickness of the gonadal layer of shallow water oysters was approximately twice that of the oysters living at a 30-foot depth.

2. The spawning of oysters at all depths began on and about July 9.

3. The shallow water oysters discharged spawn in larger quantities and more rapidly than the oysters of deeper water.

4. Among the oyster population living in shallow water there was a small group of individuals whose spawning was completed within a week.

5. Ripe-but-unspawned oysters were found in the samples collected as late as six weeks after the beginning of the spawning season. This indicates that some of the ripe oysters, living under natural conditions, cannot always be induced to spawn by a relatively high temperature and or by the chemical stimulation caused by sex products discharged by the oysters living in the immediate vicinity.

6. Approximately 50 per cent of the oyster population had half its spawn discharged by August 8. This date, therefore, is considered as the midpoint of the spawning period. Shallow water oysters reached the half-spawned stage several days earlier than the deep water individuals.

7. The spawning activities of the oyster population, living at depths ranging from 10 to 30 feet, were finished about September 3. The majority of the shallow water oysters completed their spawning somewhat earlier than those of deep water.

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THE NORMAL GROWTH AND RESPIRATION OF *TETRAHYMENA GELEII*

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The early population growth phases of *Tetrahymena geleii* (Furgason, 1940) are well established due to the work of Elliott (1933), Phelps (1935), and Hetherington (1936). The later growth phases are relatively unexplored. Phelps (1935, 1936) outlined the effect of food concentration and of the age of the inoculum. He found that Buchanan's (1918) lag phase, exponential phase, decreasing acceleration phase, and stationary phase were present in the growth of bacteria-free cultures of this ciliate and unlike most bacterial populations the stationary and lag periods were not influenced by the size of the inoculum. Varying the size of the inoculum varied only the length of the exponential phase. He demonstrated that within wide limits the concentration of food had no effect on the rate of growth in the exponential phase but only on the maximum concentration.

No complete explanation for the appearance of these various population growth phases is yet available. Food certainly plays a part, although by no means is it the only agent. Since Phelps found that in yeast autolysate concentrations between 0.4 per cent and 20 per cent there is a direct proportion between the food concentration and the maximum yield of ciliates, any factor such as Bail's (1929) "population pressure" or the presence of toxic excretory products would seem to be ruled out. Monod (1935) was likewise unable to demonstrate the appearance of any effects due to toxic substances. Kidder (1941b) suggests that an inhibitory substance as well as an accelerative factor may be produced as a result of a series of experiments on growth in "biologically conditioned" media. The oxygen tension has an effect on the length of the exponential phase and also on the maximum yield (Phelps, 1935) while the carbon dioxide tension apparently is not a limiting factor in cultures of *Tetrahymena geleii* (Jahn, 1936). The pH of the medium can influence the growth as shown indirectly by the respiration

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measurements of R. H. Hall (1941) and directly by numerous authors including Elliott (1933), Johnson (1935) and Kidder (1941a).

There has been no thorough examination of the relationships existing between population growth and cell growth in the various growth phases of protozoan cultures. These relationships become important in respiration and metabolic studies in which the information needed is the activity per unit of protoplasm rather than per organism. A study has been made therefore of the relationships existing between cell growth and population growth. This data has been correlated with normal oxygen consumption in the various population-growth phases.

It will be shown that changes in cell size may occur during the lag and early exponential population-growth phases depending on the conditions of the experiment and that consequently there may be an increase in total protoplasm due to cell growth rather than combined cell and population growth. It will likewise be shown that characteristic cell-size changes can be correlated with other population growth phases. The behavior of populations in very old cultures will be briefly noted.

This work was done at the Arnold Biological Laboratory, Brown University, and the Marine Biological Laboratory, Woods Hole, Massachusetts. I wish to express my sincere appreciation to Dr. George W. Kidder for his helpful suggestions and sustaining interest.

MATERIAL AND METHODS

The organism used in these experiments was *Tetrahymena geleii* (Hetherington strain). In the experiments on the early growth phases cultures were grown in 250 ml. Kidder culture flasks (Kidder, 1941b) in 100 ml. lots of 2 per cent Difco proteose peptone made up with Pyrex-distilled water. Cultures used in the work on size and population changes over long periods of time were grown in liter flasks using 500 ml. of culture fluid.

All cultures were grown in an incubator at $26^{\circ} \pm 0.3^{\circ}$ C. and under sterile conditions. Sterility was checked by inoculation onto nutrient agar slants and Petri dishes which were incubated at $26^{\circ} \pm 0.3^{\circ}$ C. for two weeks before a final diagnosis of sterility was made. All contaminated cultures were discarded. In the work on population and size changes over long periods the cultures were checked after each withdrawal of a sample, and at the end before the flasks were discarded.

Population counts were made by direct counting of the live organisms following serial dilution in 2 per cent proteose peptone until the concentration of the ciliates was between 25 and 125 organisms per 0.2 ml. This amount was then streaked out on glass slides and counted with a

binocular dissecting microscope. The error involved in this method amounts to ± 5 per cent.

In the work on size changes the organisms were killed by fixation in 5 per cent formalin and measured with an ocular micrometer. No appreciable shrinkage due to the formalin was observed.

Dry weight measurements were made by centrifuging the suspension of organisms, removing the supernatant and washing three times with distilled water. Ten ml. of the final suspension were then pipetted into a porcelain crucible which was placed in an oven at $100^{\circ} \pm 0.5^{\circ}$ C. for 24 hours. They were then removed to a desiccator until the weights became constant.

Nitrogen determinations of washed organisms were made by the micro-Kjehldahl method. The apparatus used is described by Pregl in Roth's (1937) translation. The method used was that of Bang (1916), Parnas and Wagner (1921) as modified by Elek and Sobotka (1926).

Respiration measurements were made with the standard Barcroft-Warburg apparatus. The technique employed is outlined in Dixon (1934). Vessels of about 10 ml. capacity were used with a total of 2 ml. of fluid in each. Carbon dioxide absorption was by means of a roll of Whatman (starch-free) No. 40 filter paper soaked with 0.2 ml. of 20 per cent KOH. The apparatus was set to give 140 oscillations per minute with a stroke of 4 cm. Temperature in all determinations was maintained at $26.8^{\circ} \pm 0.1^{\circ}$ C.

The suspensions of organisms used in respiration determinations were made up of organisms resuspended in a 0.005 M phosphate buffer of pH 6.9. The pH was checked at the end of each experiment. The density of the suspensions used was between 100,000 and 500,000 ciliates per ml. Within these limits neither the rate of shaking nor the amount of potassium hydroxide was a limiting factor in the determinations of oxygen consumption. Oxygen consumption is expressed as cubic millimeters of dry gas under standard conditions of temperature and pressure.

EXPERIMENTAL RESULTS

Normal Growth

The normal population growth curves obtained were similar to those obtained by Phelps (1935, 1936) and Kidder (1941a). Cultures were set up using 2 per cent proteose peptone in Kidder culture flasks as previously described. A normal curve (*A*) is shown in Figure 1. The lag period is not shown. The interdivisional period during exponential

growth was 3.32 hours for this set of experiments. Population counts were obtained by using as the diluent conditioned 2 per cent proteose peptone in which ciliates had been grown for the same length of time as those in the experimental flask. The organisms were killed by gentle heating in a flame, centrifuged out, and the supernatant used as the diluent. Following dilution of a sample of the experimental culture the dilutions were allowed to stand for ten minutes following which the counts were made. When a fresh 2 per cent proteose peptone was used as the

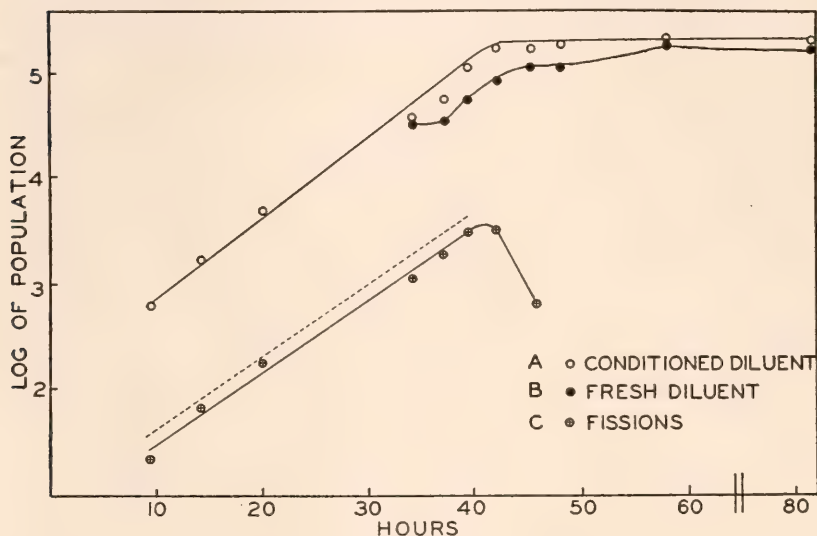


FIG. 1. Population and fission curves in 2 per cent proteose peptone. The dotted line represents the theoretical fission curve. All curves are on the same ordinate.

diluent, curve *B* was the result. This curve indicates the presence of an increased sensitivity of the organisms as the population approaches the end of the phase of exponential growth. This sensitivity was not apparent in the early exponential phase and gradually disappeared in the hours following the end of the exponential phase. It was later found that accurate counts (5 per cent) could be made using fresh proteose peptone as the diluent if the diluting and counting procedure were completed within five minutes.

The average fission time will of course vary with the criteria of the investigator. In this study fission was arbitrarily assumed to have begun when the first external indication of the cytokinesis could be observed using a dissecting binocular microscope with $10\times$ oculars and

10 $\times$ objectives. The average of 20 determinations of fission time at room temperature and in hanging drop preparations was 12.5 minutes. Curve *C* in Figure 1 represents the logarithm of the number of fissions in the exponential phase and in the hours immediately following it. The dotted line in Figure 1 represents the theoretical fission curve during the exponential phase.² As would be expected, the rate of increase of fissions is constant. The results are summarized in Table 1.

TABLE 1

Fission rate compared with the rate of population growth. Eight experiments. The theoretical number of fissions during the first 40 hours is 0.044 per cent.

Time in hrs.	Population/ml.	Fissions/ml. experimental	Per cent fissions experimental
10	594	22	0.038
14	1,585	63	0.040
20	4,740	168	0.038
34	30,600	1,060	0.035
37	53,100	1,780	0.034
40	89,100	2,982	0.034
43	141,300	3,160	0.022
46	149,700	595	0.004
49	173,800	—	—
59	211,400	—	—
82	210,000	—	—

It will be noted from Figure 1 that the percentage of fissions drops very rapidly when exponential growth ceases. After 46 hours the percentage of fissions was only 0.004. Beyond this point the number was so low that examination of 40,000 to 50,000 ciliates would not show enough fissions to form an accurate percentage figure. This indicates that in the stationary phase there is negligible death occurring. The growth rate of practically zero does not represent a balance between an increase due to fission and a decrease due to death of some organisms but an actually stationary population.

This conclusion is reinforced by a consideration of Figure 2, the data for which are contained in Table 2. Each length given in Table 2 repre-

² The population growth during the exponential phase may be described by the equation (1) $N = N_0 e^{\lambda G}$ where N = the number of individuals per unit volume, $\lambda = \log_e 2$ and G = the interdivisional period. The number of organisms at any time in the exponential phase *exclusive* of those in fission may be described by (2) $N' = N_0 e^{\lambda(G-S)}$ where S = the average time occupied in fission by each organism. Subtracting (2) from (1), and rearranging in terms of G , the percentage of individuals in fission at any time during the exponential phase is (3) $\frac{2^{S/G} - 1}{2^{S/G}}$.

TABLE 2

Cell growth compared with population growth. Size data from 14 experiments; each point represents average of 150 measurements. Weight determinations each represent average of three or more determinations. The MC signifies the end of the period of exponential growth.

Time in hrs.	Population/ml.	Length in micra	Time in hrs.	Wt. in mgm. per 10^6 organisms
0	100	74	10	5.32
4	120	68	MC	—
11	1,000	58	10	5.85
16	2,510	53	20	7.34
26	31,600	53	30	8.01
MC	—	—	40	—
3	123,200	57	50	8.34
19	135,000	70	60	8.79
26	158,600	73	70	8.82
40	162,500	75	80	—
75	174,300	75	90	8.85
100	128,800	75	130	8.55 ^a
130	120,000	75	191	9.04 ^b
145	102,700	71	—	—
169	100,100	70	—	—
191	89,200	69	—	—

^a One determination.

^b Two determinations.

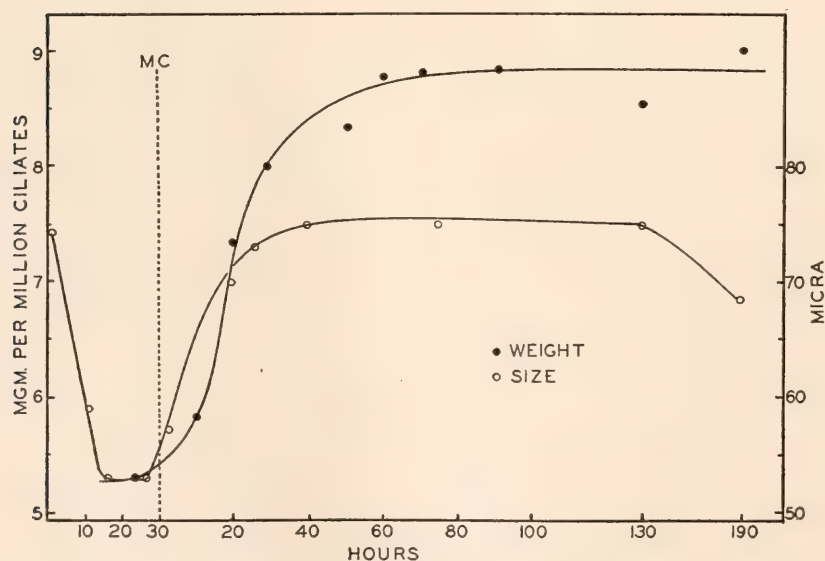


FIG. 2. Size and weight changes in 2 per cent proteose peptone flask cultures of *Tetrahymena geleii*. The abscissa represents the age of the culture.

sents an average of 150 measurements. The length was taken as an adequate criterion of the size, since the ratio of length to width did not vary significantly over the time range examined. The weight measurements are the average of 36 determinations and are plotted as the average of 10-hour intervals. Data for both weight and size are translated into terms of a 30-hour exponential period for purposes of comparison, and all determinations made after the exponential phase were calculated with the end of that period as the point of departure. Data such as these are significant in terms of the age of the culture with respect to the growth

TABLE 3

Nitrogen determinations. Three determinations were made on urethane-treated organisms in a separate experiment not reported here.

Dry wt. in mgm. of organisms	N in mgm.	Per cent N	Age of organisms in terms of 30 hour MC
27.1	3.1	11.4	23 hours
67.5	4.8	7.1	84 hours
62.6	6.5	10.4	93 hours
64.6	7.3	11.3	110 hours
22.1	2.2	10.0	127 hours
27.3	2.9	10.6	40 hours
12.9	1.3	10.8	47 hours ^a
14.0	1.6	11.4	34 hours ^b
71.7	7.7	10.7	87 hours
12.4	1.4	11.3	59 hours ^c
	Average	10.5	

^a 13 hrs. in 1 per cent urethane.

^b 11 hrs. in 1 per cent urethane.

^c 25 hrs. in 1 per cent urethane.

phases and not in terms of absolute time units. With different-sized inocula the length of the exponential phase in these experiments varied from 15 to 45 hours. All weight measurements made on exponential phase organisms, when translated into terms of a 30-hour exponential growth period, came at the same place in time, e.g., 25 hours. Since they all agreed very closely it is clear that the weight of the individual ciliates is quite constant during the exponential phase. The time relations in the increase in size and weight following the end of the period of exponential population growth were comparable, regardless of the length of the exponential period. This demonstrates that size and weight measurements on organisms such as these must be judged with relation to the normal population curve and not by an absolute time scale.

Nitrogen determinations (Table 3) indicate a constant nitrogen per-

centage of 10.5 per cent. In other words, the increase in weight shown in Figure 2 represents an increase in protoplasm and not merely an increase in stored carbohydrate or something of like nature. The average nitrogen content agrees well with the scanty data available. Panzer (1911), in an analysis of the coccidian *Gaussia gadi*, found the nitrogen to be 9.7 per cent of the dry weight. Hutchens (1941a) reports the nitrogen content of *Chilomonas paramecium* to be 10 to 11 per cent.

In the lag and early exponential phases the shape of the size curve in Figure 2 depends upon the size of the ciliates used for inoculation of the culture. If organisms 50 to 75 hours old are used they are usually about 75μ long. Upon inoculation into fresh media and the onset of division, the average size progressively decreases until a constant size of around 53μ is reached. This size is then maintained to the end of the exponential period. On the other hand if organisms from a 30- to 40-day old culture (about 35μ long) are used for the inoculation, there is an increase in average size until the constant size of about 53μ is reached. Thus it is clear that in some cases at least the changes in individual size preceding and during cell division must be interpreted in relation to the age of the organism.

Population changes were followed for periods up to 90 days and cell-size changes up to 45 days in 2 per cent proteose peptone cultures. In cultures in which the MC occurred at 36 hours, the stationary phase of population growth persisted for five days, following which a phase of declining population set in. This phase was sometimes marked by rather sudden increases in population although the overall picture was one of gradually decreasing population. It is clear that the latter parts of Buchanan's classical population curve are not present in cultures of this type and in the time range examined. There is no clear phase of accelerated death or exponential death and the population decrease that is observed is not necessarily an even decline.

Cell size in cultures of this type, after increasing to a maximum of 75μ in length as shown in Figure 2, maintained the maximum size for four days after which there was a gradual decrease. In three experiments this decrease in size continued for 34 days at which point there was no further change for six days when the experiments were discontinued. The ciliates had decreased to 36μ length in these 40-day old cultures.

Normal Respiration Rates

Table 4 gives the results of normal respiration measurements on ciliates in 0.005 M phosphate buffer and in a 2 per cent proteose pep-

tone medium buffered with 0.005 M phosphate. The Q_{O_2} is defined as cu. mm. O_2 /hr./mgm. dry weight of tissue. The exponential phase ciliates were taken between 20 hours and 30 hours from cultures in which exponential growth would have ceased at 30 hours. The stationary phase organisms were taken at around 50 hours from similar cultures.

Provided the concentration of organisms in the suspension measured is above the maximum concentration obtained in a normal culture, the

TABLE 4

Normal respiration values. The figures in parentheses refer to the number of determinations made. The Q_{O_2} represents cu. mm. O_2 /hr./mgm. dry weight of ciliates.

	Exponential phase organisms		Stationary phase organisms	
	In 0.005M PO_4 buffer (65)	In 2% proteose peptone (11)	In 0.005M PO_4 buffer (21)	In 2% proteose peptone (14)
Q_{O_2}	26.0	81.1	16.5	78.8
Cu. mm./hr./ 10^6 ciliates.....	140.2	433.0	133.7	633.0

oxygen consumption is a linear function of time over a period of three hours in all cases. Experiments were terminated at three hours.

It might be expected that exponential phase ciliates would show a higher rate of respiration than organisms in the stationary phase. This difference does appear, particularly in buffer suspensions.

DISCUSSION

Very little satisfactory work correlating population with size changes has been done with protozoa although there are good data on bacteria and yeasts. Henrici (1928) showed that cells of *Bacillus megatherium* increase in size during the lag period and up to the point at which population growth rate is maximum, whereupon they begin to decrease in size immediately. Huntington and Winslow (1937), in an examination of the size changes in *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum*, show that in all three there is an increase in size in the lag period and a decrease in size during the exponential period. Richards (1928) has shown that yeast cells increase in size in the very early growth period and then decrease in size during the late exponential growth to a size comparable with that of the cells originally inoculated. The observations on protozoan populations have largely been made on

cultures contaminated by bacteria. Maupas in 1888 observed that individuals decreased in size during "senile degeneration" in old cultures. Calkins (1904) likewise showed that *Paramecium* decreased in size as the cultures died out. Woodruff's (1913) data on the growth of *Oxytricha fallax* can be roughly correlated with the growth phases. He observed that individuals were small during rapid growth and increased in size later. Peters (1921), on the other hand, noted that in cultures of *Colpidium colpoda* the population grew very slowly for nine to ten days during which time the individual size increased and remained large during the period of rapid growth which followed. Later the decrease in size was correlated with a decrease in population. Vieweger (1925) states that in cultures of *Colpidium colpoda* the body size decreased following the period of rapid exponential growth. His first measurements following the end of the exponential phase appear to have been made at six days, however.

All the work mentioned in the preceding paragraph was done with contaminated cultures and without complete data regarding the population curve being obtained. Bond (1933), using sterile cultures of *Tetrahymena geleii* (*Colpidium campylum*), and Lwoff (1932), using *Tetrahymena geleii* (*Glaucoma piriformis*), give data that indicate an increase in individual size following the exponential period. Their data concern total dry weight and total nitrogen change respectively. Dewey and Kidder (1940) give complete data on change in size in *Perispira ovum* fed on *Euglena gracilis*. They show an increase in size in the lag and early exponential phases. The size changes in *Perispira ovum* cannot be compared directly to those in *Tetrahymena geleii* since the increase in size in the former may be due largely to the accumulation of unassimilated *Euglena* in the individual ciliates. The subsequent decrease in size beginning in the late exponential period is correlated with a drop in the concentration of the food organism, *Euglena*. Any change in individual amount of *Perispira* protoplasm is obscured by the presence of masses of ingested but unassimilated *Euglena*.

The present investigations have brought out several points with regard to normal growth in populations of *Tetrahymena geleii*. The stationary phase of growth, so-called, has been found to be a truly stationary phase only so far as the numbers of individuals are concerned. It is seen that the number of fissions in this stage is very low while the population remains essentially static in number. This indicates that the individual organism has a much longer life in this phase and that there is very little death occurring. It is also seen that another process, that of individual growth, proceeds concurrently with that of population

growth until the end of the exponential phase whereupon it continues with the result that in the hours immediately following the onset of the stationary phase the organisms increase in size. It is thus apparent that the population curve taken alone is not a true representation of the growth of the culture.

Normal respiration data on *Tetrahymena geleii* is summarized in Table 5. M. Lwoff (1934) obtained the figure of 35 cu. mm. O₂/hr./mgm. dried organisms in peptone water using the Warburg method but does

TABLE 5
Comparative values of QO₂ for Tetrahymena geleii.

Investigator	Approx. age of culture	Bacteria-free	Temp. °C.	Substrate present	Cu. mm. O ₂ /hr./10 ⁶ organisms	Cu. mm. O ₂ /hr./mgm.
Peters (1929) ^a	?	Yes(?)	?	+	50-200	6.2-24.7 ^b
M. Lwoff (1932).....	?	Yes	22°	+	271.5 ^b	35
Baker & Baumberger (1941)	48 hrs.	Yes	20°-22°	+	154 ^b	19.01
Pitts (1932).....	48 hrs.	No	24°	-	151	18.7 ^b
R. H. Hall (1938-1941).....	48 hrs.	Yes	19.8°	+	112.5	13.9 ^b
Present investigator.....	50 hrs.	Yes	26.8°	-	133.7	16.5
Present investigator.....	50 hrs.	Yes	26.8°	+	632.5	77.7

^a The ciliate used was called *Colpidium colpoda*. It may or may not have been *T. geleii*.

^b These values are computed on the basis of the weight curve in Figure 2. 10⁶ organisms equal 8.1 mgm. at 50 hrs.

not mention the age of the organisms used or the number of organisms per mgm. of dry weight. Pitts' (1932) figure was obtained using a non-nutrient suspension that had grown on bacteria for 48 hours previously. Baker and Baumberger (1941) used a dropping mercury electrode and a suspension of *Tetrahymena geleii* in yeast autolysate dextrose. They did not determine the dry weight but calculated it on the basis of 50 μ being the average length of an organism and the dry weight being about 15 per cent of the wet weight. R. H. Hall (1938, 1941) apparently used M/15 Sørensen phosphate and 0.5 M Clark and Lubs biphthalate buffers for his suspensions. It was found in the course of these investigations that M/50 Sørensen phosphate buffer depressed the respiration rate markedly and that even M/100 phosphate had a slight inhibitory effect.

A number of factors may have contributed to the different results shown in Table 5. The organisms in the several cases may not have been of comparable age and weight and there may have been consider-

able strain differences. Likewise there may have been some errors due to bacteria or the products of bacterial metabolism.

The age of 48 hours is significant only when the length of the exponential phase is known likewise, so the figures given in Table 5 on the age of the cultures are for the most part only rough approximations of the actual age of the ciliates in terms of the population curve. Strain differences are known to exist and may exert an influence on the rate of respiratory activity. The possible toxic or beneficial action of bacteria is well known. There may have been errors in the determinations by Baker and Baumberger due to their approximations regarding volume and weight of the ciliate. The inhibitory action of phosphate buffer solutions previously mentioned may vitiate Hall's figure. The different substrates used in the various determinations are undoubtedly influencing factors. The most probable reason for the high Q_{O_2} obtained in the present investigation is the utilization of a non-toxic concentration of buffer, and a substrate that is comparatively concentrated and to which the organisms have been thoroughly acclimated.

In comparing the rate of respiration in exponential and stationary phase ciliates in buffer suspensions on the basis of number of individuals, the values obtained were 140.2 cu. mm./hr./ 10^6 organisms and 133.7 cu. mm./hr./ 10^6 organisms respectively. When buffered 2 per cent proteose peptone suspensions were used the rate per 10^6 organisms is higher in the *stationary* phase. This situation is clarified when reference is made to Figure 2 and Table 4 and illustrates the errors that may be involved in comparing respiration rates on a population basis alone. The Q_{O_2} is defined as the cu. mm. O_2 /hr./mgm. dry weight of respiring material. On this basis the Q_{O_2} of exponential phase ciliates is 26.2 since the weight of 10^6 organisms is 5.33 mgm. As the weight of the stationary phase ciliates increases rapidly following the end of the exponential period, the weight of 10^6 organisms at 50 hours is 8.10 mgm. This gives a Q_{O_2} of 16.5 or 63 per cent of the rate found in the exponential phase. This emphasizes the point that respiration rates should be compared on the per-unit-of-protoplasm basis rather than on the basis of individual organisms. The relatively greater increase in Q_{O_2} in stationary phase organisms when in proteose peptone suspensions suggests that the conditions under which measurements are made are much more like those of stationary phase cultures than those of exponential phase cultures due to the high concentration of organisms. External conditions may be limiting factors in the respiration of exponential phase organisms under these conditions.

In the matter of decreasing oxygen consumption with increasing age of the culture there is unanimity. Peters (1929) reports a drop from a Q_{O_2} of 200 in growing cultures of *Colpidium colpoda* to one of 50 in old cultures. Wachendorff (1912) reports a like decrease in the respiration of *C. colpoda* within 30 days from the time the culture was started. Baker and Baumberger (1941) show a decrease in *Tetrahymena geleii* suspensions beginning in the last exponential phase and dropping to approximately one-third the highest value obtained during that period, in from three to seven days. Hutchens (1941b) has found the same phenomenon in *Chilomonas paramecium* within a period of 72 hours. In the present investigations, likewise, a decrease in oxygen consumption has been found in the population phases following the exponential period of growth.

SUMMARY

1. The relation of cell growth to population growth in bacteria-free cultures of *Tetrahymena geleii* has been examined.
2. Cell growth has been found to proceed independently after the onset of the stationary period of population growth.
3. The ratio of fissions occurring to the population concentration has been determined and described.
4. The normal Q_{O_2} of exponential and stationary phase ciliates in phosphate buffer and in proteose peptone has been determined.
5. A significant difference in the respiratory rates of organisms from the two phases has been shown.

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THE SEXUAL CYCLE OF THE SHIPWORM, *TEREDO NAVALIS*

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In an earlier paper Grave and Smith (1936) published results of an extensive study of adult *Teredo* (over-wintering *Teredo*) which showed conclusively that numerous individuals change sex, not only from male to female but also from female to male, during the course of the breeding season. That this is a part of the normal sexual cycle of *Teredo* is shown by three types of evidence: a study of stained paraffine sections; teased gonads of living animals; and a tabulation of sex ratios.

During the past summer a similar study was made of young shipworms of the current season in the region of Woods Hole with comparable and confirmatory results. Early in the summer *Teredo* traps in the form of wooden crates made of lobster pot lath or of fir boards one-half inch in thickness and approximately three inches wide were placed in sea water known to be infested with *Teredo* larvae. By examining slats from these traps at frequent intervals during the summer it was possible to follow the course and rate of development of the young animals. More important for the purpose of this study, it was possible to determine the age at which they first became sexually mature and to tabulate sex ratios at various stages of development consecutively. Table I therefore shows the approximate age of the animals examined, the increase in size with age or rate of development, and the relative numbers of the sexes from week to week during the breeding season.¹

¹ Sexually mature males are easily recognized. When removed from their burrows they expel active spermatozoa visible under a low power of the microscope. In practice it was found necessary to place each individual in a separate dish and the test of maleness was the presence of abundant active sperm in the surrounding water. As a further test the body wall was torn with dissecting instruments and a portion of the gonad removed and examined microscopically to determine its character, whether male, female, or sexually immature. If male, abundant active spermatozoa were released; if female the gonad wall showed a complete layer of large yolky oögonia. Sexually mature females are characterized by oöcytes which readily separate from the gonad wall when teased with needles. It should be noted that most males have a few or numerous small oögonia without yolk (Grave and Smith, 1936, Plate II, Figures 5 and 6). The entire gonad of both male and female with its ducts resembles a bunch of grapes except that the terminal alveoli which produce the germ cells are not spherical but elongated, sinuous, and somewhat intertwined.

The points of chief interest are that some of the young animals became sexually mature at 40 or 42 days when they measured from 27 to 30 mm., and that males and females appeared simultaneously. This rate is the most rapid attained at Woods Hole. Larvae which metamorphose late in June or early in July require seven or even eight weeks to reach sexual maturity.

Although only a few individuals are sexually mature at this early stage the data show that males and females appear simultaneously. The data of Table I dealing with somewhat later stages of growth show that throughout the breeding season there is no great preponderance of one sex over the other. It is only toward the end of the first breeding season that the females appear in considerable excess and that excess never approaches 90 per cent either in young or in over-wintering animals. This is in substantial agreement with other tabulations of sex ratios in *Teredo* (Grave, 1928; Grave and Smith, 1936; Coe, 1936).

Statistical tabulations are always open to criticism because of the unavoidable element of analysis and of conscious or unconscious manipulation. It is therefore desirable to bolster such studies, valuable as they may be, with facts or observations not subject to more than one interpretation. If it can be shown that female teredos are changing to males during the entire breeding season it should be evident that the population could not become 90 per cent or exclusively female in adult life as required by the theory of protandry. The following examples which are described in detail are of frequent occurrence. They constitute the chief evidence that this sex inversion is a normal part of the sexual cycle of *Teredo*.

EVIDENCE OF CHANGE OF SEX FROM FEMALE TO MALE

There is dependable evidence that *Teredo* may and does change sex during the breeding season. It is not in question that a sexually mature male may change to a female. That individual teredos also change from female to male is supported by observations of specific cases, three of which are presented. These cases, it is believed, are subject to no other interpretation. The so-called hermaphroditic teredos are not really hermaphroditic but are transition stages of brief duration of individuals in process of changing from male to female or from female to male so that the male and female phases may overlap for a day or two. This point cannot be too strongly emphasized and any investigator who studies *Teredo* extensively is likely to come to this conclusion. Nearly all individuals are either in the male phase exclusively, or exclusively female and not both. The examples selected will serve to corroborate this interpretation of hermaphrodites.

Case I.—A young *Teredo* of the current season two months old was carrying large numbers of veliger larvae in its brood pouches. From their color the veligers were judged to be ten days or two weeks old. In other words, this individual had been a female when the eggs producing this brood of larvae had been spawned. An examination of its gonad showed it to be a testis containing great numbers of active spermatozoa and spermatogenous tissue. In the wall of the gonad there were also a few small quiescent yolkless oögonia. This is a characteristic of *Teredo* in the active male phase (Grave and Smith, 1936, Plate II, Figures 5 and 6). It is noteworthy also that this was a young *Teredo* which changed from female to male after a single spawning. It was not old enough to have spawned twice since it could not have become sexually mature before six weeks of age. Point is made of this case because it shows that females are changing to males during the first season as well as in the adults which were described in detail and with illustrating photographs in a former paper (Grave and Smith, 1936).

Among cases of sex inversion from male to female observed in young teredos during the past summer, one was two months old, another ten weeks. Since they are not otherwise significant to this study they are not described and the reader is referred to published descriptions and photographs (Grave and Smith, 1936, Plate II, Figure 4) which show how a male changing to a female may be recognized.

Case II.—During the present study of adult shipworms numerous cases of sex inversion from female to male were observed and examples were selected for description. One of these showed the following characteristics: It contained numerous veliger larvae and also abundant cleaving eggs in its gills, showing that it had spawned twice as a female. Its gonad contained abundant active sperm and no oögonia. There can be no doubt that this individual had recently changed sex from female to male.

Case III.—Stained paraffine sections prepared for the study of gonads of adult shipworms showed all degrees of maleness and femaleness, including true males. From hundreds of preparations one individual changing from female to male was selected for description, a photomicrograph being made to record its character. Each of the alveoli of the gonad contained one or more oöcytes free in the lumen while the parietal wall was clearly composed of spermatogenous tissue, including spermatogonia, spermatocytes and spermatids. This is clearly the condition of the gonad because abundant spermatozoa were already free in the lumen surrounding the free oöcytes. This individual might be called an hermaphrodite but no oögonia were present in the gonad wall

and as soon as the floating oöcytes had passed out it would have become exclusively male. This photograph can be interpreted in no other way than that the individual was changing from female to male (Grave and Smith, 1936, Plate I, Figure 3).

It has been the purpose of the foregoing account to present positive evidence from the study of living animals and paraffine sections that teredos normally and regularly change sex, beginning shortly after they

TABLE I

Rate of growth and relative numbers of the sexes of young Tereido taken from Tereido traps composed of small fir boards placed in sea water at Woods Hole, July 1, 1941

1	2	3	4	5	6	7	8
Date of collection	Approximate age	Size of burrows	Males	Females with eggs	Females with embryos	Hermaphroditic	Not spawning
Aug. 8	38 days	8-10 mm.					5
" 9	39 "	8-12 "					10
" 10	40 "	16-20 "					6
" 11	41 "	16-22 "					10
" 12	42 "	20-22 "					2
" 19	49 "	28-35 "	4	1			1
" 19	49 "	40-43 "	1	2	2		
" 21	51 "	44-52 "	4	1			
" 22	52 "	35-39 "	2	1			1
" 22	52 "	40-60 "	5	6	7		
" 23	53 "	40-62 "	5	3		1	
" 28	58 "	40-45 "	6	2	1	1	1
" 28	58 "	50-70 "	4	4	8	2	1
Sept. 10	72 "	60-85 "	4	2	16		10
" 13	75 "	50-75 "	2	6	1		2
" 13	75 "	80-130 "	14	8	14	1	10

Table I, when read from top to bottom, shows in column 1 the successive dates of collection of *Tereido* from crates exposed to sea water on July 1; column 2 gives the maximum age of the specimens collected (by selecting only the largest specimens it may be assumed that they are approximately as old in days from metamorphosis as indicated in column 2); the size of the burrows as shown in column 3 is considered to be an accurate measure of size attained, since the animal in life exactly fills the burrow when relaxed; columns 4, 5 and 6 show the relative numbers of males and females found on successive collections; column 7 includes a small number of individuals whose gonads contained both active sperm and fully developed yolky oöcytes; column 8 includes all individuals which contained neither active sperm nor recognizable oöcytes which would indicate an approach to sexual maturity. However, the last three items of column 8 were dead animals which had disintegrated beyond recognition of sex, indicating a high mortality after September 1.

Table I not only shows the relative numbers of the sexes at successive periods of the breeding season but also indicates that teredos in the progonad stage are not sexually mature. (See first five items of Table I.) This conclusion is based upon the fact that no active sperm and no eggs are expelled during this early period.

become sexually mature and continuing throughout the remainder of their lives. This inversion of sex is in both directions, from male to female and from female to male. It has been shown (Grave and Smith, 1936) that females change to males not only at the opening of the second breeding season, as described by Coe, 1934, but throughout the breeding season of adult or over-wintering teredos.

The investigations of the past summer, reported in this paper, show that the inversion of sex begins in animals only two months old and continues thereafter to be a part of sexual history of the species. It is clear therefore that if there is at all times a considerable inversion of females to males the sexual cycle of this species could not be described as overwhelmingly male in young animals and equally overwhelmingly female in mature animals. This type of sexual cycle has been attributed to *Teredo* and referred to as protandric. This study shows that both sexual changes occur throughout the breeding season, and consequently *Teredo* resembles the California oyster rather than *Crepidula*, both of which have been fully described by Coe. This contribution is considered important not merely because it adds a point to scientific accuracy but because of its bearing upon the problem of sex determination. Scientific literature dealing with sex determination when considered as a whole, especially that dealing with insects, mammals, and molluscs, is confused and it is idle to talk about a mechanism of sex determination. It is obviously more difficult to account for a mechanism of sex inheritance in an animal that changes sex three or four times than in one that is male when young and female at maturity (protandric). If investigators will keep facts clearly separated from interpretation and use principally either photographs or camera lucida drawings for illustration instead of diagrams, some future biological theorist may be able to unify all of the numerous and diverse contributions to the study of sex and sex inheritance. Sex changes in molluscs have been induced or hastened by changes in the environment of various kinds but internal factors are obscure. This has been shown to be true, especially by the experiments of Coe on *Crepidula*.

THE PROGONAD AND THE SEXUAL CYCLE

This account to this point has not involved the very early stages in the development of *Teredo*, which precede sexual maturity. Young shipworms measuring from 10 to 20 millimeters and estimated to be not more than four to five weeks of age were fixed, sectioned and stained for microscopic study of the protonad.

At an early stage of development the gonad of *Teredo* consists of a solid mass of undifferentiated cells lying on either side of the digestive tract. In the process of further development it becomes a branching system of tubules with walls several cells in thickness but as yet indistinguishable as to sex. Following upon this, in some individuals, certain cells enlarge and become recognizable as oögonia. These large cells arise in the midst of more numerous smaller cells which still appear to be undifferentiated. Whether the latter are actually undifferentiated or are potentially spermatogonia and oögonia is difficult to make out. In any case individuals which have large oögonia in the gonad wall at this early stage of development are destined to become females, either purely female or with some spermatogonia among the developing oögonia. These oögonia early take on a deposit of yolk and the spermatogonia, if present, become aborted without functioning, and the gonad becomes an ovary. Other individuals are destined to differentiate into males whose gonads appear to be purely male or they may contain small yolkless oögonia which remain quiescent for the time being. These individuals produce abundant active sperm and are functional males. Thus the cytological evidence confirms results obtained by the study of living animals to the effect that males and females develop simultaneously. Both males and females appear in considerable numbers within eight weeks of metamorphosis. One gets the impression that the balance between femaleness and maleness in *Teredo* is not rigidly fixed at any period of its development so that males may become females and females become males with slight alteration of internal or external conditions not as yet understood. The young gonad appears to be indifferent as to sex and as a result all gradations between maleness and femaleness appear in the gonad itself.

Although the progonad does not produce active spermatozoa it, in a sense, may be considered a male phase of brief duration. This would be true only if the undifferentiated cells in the young gonad surrounding the developing oögonia are properly considered to be spermatogonia. Otherwise approximately half of the young teredos are actually female. In any case numerous individuals differentiate first as functional females and do not produce active sperm. It has been estimated that about half of the individuals are females from the first appearance of germ cells in the young gonad.

DISCUSSION AND CONCLUSIONS

The sexual cycle of *Teredo* may be regarded as beginning with the differentiation of sex. By the end of eight weeks nearly all individuals are sexually mature and the inversion of sex from males to females and

from females to males begins. This change of sex from female to male prevents any *Teredo* population from becoming overwhelmingly of one sex either at maturity or at the beginning of sexual life. Both sexes are well represented at the end of eight weeks of age and because of the inversion of sex in both directions both sexes remain well represented in all tabulations of sex ratios that have been made. It should be clear therefore that the observed changes from female to male during the breeding season constitute part of the sexual cycle in this species.

It remains only to point out that the term "protandric female" as used by Coe (Coe, 1933) is synonymous with the term "functional male" or "bisexual male" as used by Grave and Smith, 1936, and in this paper. The writer prefers to regard such individuals as males since they constitute most if not all of the existing males, while Coe considers them potential females in anticipation of their change to females as a part of a protandric sexual cycle. If they actually do change to females without any counterbalancing change of females to males the population would ultimately become wholly or predominantly female. Since it has been shown that females do change to males there is necessarily a recognition of the fact that the general population remains always considerably male, and the conception of a protandric sexual cycle should be modified to recognize this feature, emphasizing the fact that there are changes of sex in both directions during the entire breeding season as well as at its beginning.

Since the completion of this manuscript, September 20, 1941, an article by Coe (1941) has appeared. As in former papers, he insists on the essentially protandric nature of the sexual cycle of *Teredo navalis*. His facts and mine are similar except that I do not consider the progonad as 90 per cent male. The point made in this paper, considered to be its chief contribution, is that the changes in sex during the breeding season are not exclusively from male to female as required for a protandric sexual cycle but also from female to male. It has been pointed out that this latter sex inversion necessarily changes the whole concept of the sexual cycle and accounts satisfactorily for the high percentage of males present during the entire breeding season. Coe regards the succession of sex changes from male to female as a function of age and he therefore considers the general population as being composed of animals of many different ages, thus accounting for the persistence of numerous males at all times during the entire course of the breeding season.

In a former paper the writer showed that all over-wintering teredos, even those that are too small to be detected in the wood during the winter months, become sexually mature before the end of June (Grave, 1928).

The over-wintering teredos are therefore not just becoming sexually mature all summer. It would seem also that the observed changes from female to male which take place in both young and over-wintering teredos should be considered to be an essential part of the sexual cycle. It is clear also that a considerable number of individuals function first as females and these have no primary functional male phase. *Teredo navalis*, therefore, according to this interpretation, is not essentially or principally protandric.

I wish to express my obligation to Dr. J. A. Smith for assistance in embedding and sectioning material and for criticizing the manuscript.

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THE INACTIVATION OF FERTILIZIN AND ITS CONVERSION TO THE "UNIVALENT" FORM BY X-RAYS AND ULTRAVIOLET LIGHT

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Recently Tyler (1941) has shown that the substance known as fertilizin which is obtained from the eggs of certain marine invertebrates and which has the property of agglutinating species sperm, may exist in non-agglutinating form. Thus, fertilizin (egg water) of *Strongylocentrotus purpuratus*, when subjected to appropriate heating or enzymatic digestion, lost its ability to agglutinate, but retained its ability to combine with the sperm. Tyler has proposed the term, "univalent fertilizin," for such non-agglutinating substances. They are readily demonstrable since their addition to sperm inhibits subsequent agglutination by ordinary fertilizin of the same species.

Several workers have shown that the sperm agglutinating power of *Arbacia* egg water can be destroyed by X-rays. Richards and Woodward (1915) reported an initial rise and subsequent fall in sperm agglutinin titer with prolonged irradiation. More recently, Evans (1940), and Evans, Beams and Smith (1941) have shown that the egg jelly of *Arbacia* is dissolved by X-rays and that irradiated egg water will not give the Janus green reaction (Harvey, 1939) nor will it agglutinate species sperm.

It appeared possible that univalent fertilizin might be produced during treatment of fertilizin with X-rays. The present work was done primarily to examine this possibility. The results show that it does indeed occur. In the course of the work further quantitative data were obtained on the X-ray inactivation of fertilizin in terms of Roentgen units, fertilizin concentration and sperm concentration. In addition the possible action of another type of radiation, namely ultraviolet light, in inactivating fertilizin and producing univalent fertilizin was investigated. Here, also, the results were positive.

MATERIAL AND METHODS

The sea urchin, *Arbacia punctulata*, was used exclusively in the experiments with X-radiation, while *Strongylocentrotus purpuratus* was

used in the experiments with ultraviolet light. Eggs and sperm were obtained by opening the animals and allowing them to shed into stender dishes. Fertilizin was prepared by removing the supernatant sea water from a suspension of eggs which had stood overnight in the refrigerator, or by extracting eggs in acid sea water (Tyler and Fox, 1940) and readjusting the pH of the extract. All sperm suspensions were made from "dry sperm" (sperm collected from the gonopores in dry stender dishes). The concentrations of sperm are given as percentages of this dry sperm. The presence or absence of an agglutination reaction was determined by microscopic examination two to four seconds after mixing the solutions and sperm suspensions.

X-radiation in all cases was done in waxed paper cups at 5,600r/minute.

Ultraviolet radiation was given in open stender dishes. Controls were run in open dishes placed at the same distance (4.5 cm.) from the radiation source, but screened by a filter (Noviol "C"). A quartz-mercury arc (tube dimensions: 56×0.8 cm., power supply: a 15,000 volt, 30 milliamperes transformer) was used as the ultraviolet light source.

EXPERIMENTS

Inactivation of Fertilizin by X-radiation

In a typical experiment, a sample of egg water obtained as the supernatant from 50 cc. of eggs was divided into five equal parts. One part was kept as control and the other four samples were irradiated as indicated in Table I. After irradiation the agglutinating titer of each sample

TABLE I

Destruction of agglutinating power of Arbacia egg water by X-radiation

r units	Fertilizin titer	Per cent inactivation
0	2,048	—
2,800	1,024	50
5,600	256	87.5
16,800	2 (?)	99.9
28,000	0	100

was determined by serially diluting the samples in $\frac{1}{2}$ dilutions with sea water and testing each dilution with an equal amount of 0.5 per cent sperm suspension. Titers are given as the greatest dilution at which agglutination is still microscopically perceptible. The units are, there-

fore, roughly 20 to 40 times greater than those employed by Tyler and Fox (1940).

As shown in the table, inactivation of this sample of fertilizin (titer 2,048) is practically complete with irradiation above 16,800r. Two other experiments gave similar results.

Richards and Woodward gave no information concerning the output of their X-ray apparatus. Since they used a very weak fertilizin solution and noted only a slight fall in agglutinin titer after 7.5 minutes of irradiation, it may be concluded that they used relatively low X-ray doses. Evans, Beams and Smith (1941) irradiated samples of *Arbacia* egg jelly (egg suspensions?) with 61,000r and 122,000r. The former sample gave light agglutination and the latter gave none. These values are in agreement with those presented here, assuming that dilute test sperm suspensions were used, since the same workers have shown, in *Arbacia*, that X-ray doses of 15,000r completely remove the jelly, which is identical with or contains the fertilizin (Tyler, 1940, 1941). Thus, an irradiated egg suspension is equivalent to an irradiated fertilizin solution of high titer.

Production of Univalent Fertilizin by X-Rays

As is well known (Lillie, 1919; Tyler, 1940), the agglutination of sea urchin sperm by egg water is a spontaneously reversible phenomenon. Furthermore, sperm which have been agglutinated by a sufficiently strong egg water will not, after reversal, reagglutinate upon further addition of fertilizin. Theoretically, sperm treated with fertilizin that had been converted to the univalent form should be equivalent to sperm that had reversed and so no agglutination should occur upon subsequent addition of normal fertilizin.

The sperm agglutinin titer of any fertilizin sample may be determined by serially diluting the sample and testing for agglutination with constant amounts of a sperm suspension, as previously outlined. If, after reversal of this agglutination, equal amounts of a test (normal) egg water are added to each dilution and the greatest dilution at which no agglutination occurs is recorded, a quantitative measure of the agglutination inhibiting power of the sample is obtained, which may be termed the inhibition titer of the sample. When both the agglutinin and inhibition titers of an irradiated sample and its unirradiated control are determined, using the same sperm suspension and test egg water solution, the formation of any univalent fertilizin through the treatment may be detected by a comparison of these values. It is thus not necessary to inactivate the fertilizin completely to test for the univalent form. This is desirable, in

that excessive irradiation might result in complete destruction of the fertilizin.

A strong fertilizin solution was prepared by extracting eggs in slightly acid sea water (pH 4.5). By this treatment 105 cc. of egg suspension gave 65 cc. of egg water and 40 cc. of jellyless eggs. After readjusting the solution to pH 8, a 5 cc. sample was removed and irradiated for five minutes at 5,600r/minute. Two drops of the irradiated solution and two drops of the control were diluted serially in tenfold steps with sea water, two drops of a 1 per cent sperm suspension were added to each dilution, and the dishes were allowed to stand until the agglutination in the undiluted control had reversed. Two drops of 1/10 dilution of the original control fertilizin were added and the dishes examined for agglutination.

TABLE II

Ability of X-rayed Arbacia fertilizin to react with sperm and thus inhibit subsequent agglutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of a test egg water to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control egg water	100,000	Negative	1,000
Irradiated egg water . . .	100	Negative	1,000
1,000 fold dilution of control egg water . . .	100	Positive	0

The irradiated egg water was found to have about 1/1,000 the agglutinin titer of the control. However, it was found to be just as effective as the control solution in preventing subsequent agglutination. The quantitative data are given in Table II.

It is of interest that no precipitate which might be attributed to this radiation was seen to form in this or any of the other X-rayed fertilizin solutions, even after prolonged exposure.

Inactivation of Strongylocentrotus Fertilizin by Ultraviolet Light

A stock solution of *Strongylocentrotus purpuratus* fertilizin was made up by extracting, with acid (pH 3) sea water a concentrated suspension of shed eggs, centrifuging and adjusting the supernatant to pH 8. Two 4 cc. samples were removed from this stock solution. One was irradiated and the other used as a control as previously described.

At various intervals of time equal portions of solution (four drops) were removed from the irradiated and control samples for titration of

sperm agglutinin activity. Titrations were made by adding equal amounts (two drops) of 1 per cent sperm suspension to equal amounts of the fertilizin diluted serially in $\frac{1}{2}$ dilution steps. The titer, as in the case of *Arbacia*, was taken as the greatest dilution at which agglutination could be observed in two to four seconds under the microscope. Since the same 1 per cent sperm suspension, pipettes, etc., were used throughout, the titrations, as presented in Table III, are directly comparable. The results are typical of several experiments.

TABLE III

Destruction of agglutinating power of S. purpuratus fertilizin by ultraviolet light

Duration of irradiation	Agglutinin titer	Per cent inactivation
0	16,384 ¹	—
60 min.	1,024	93.8%
90 "	256	98.4%
124 "	16	99.9%
154 "	0	100.0%
349 "	0	100.0%

¹ Average of the five controls, none of which differed more than one dilution step from this value.

As the data indicate, complete inactivation of the sperm-agglutinating property of this fertilizin solution was attained in 2.5 hours by the ultraviolet light source. Since both the controls and irradiated samples were allowed free access to air and were the same distance from the radiation source, evaporation and effect of ozone are ruled out as causal agents. Thermometers placed in the position of the control and irradiated samples indicated at most a 2° C. rise above room temperature, but never a difference of more than 1° C. in the two positions, so the inactivation found is attributed to the radiation from the arc.

Production of Univalent Fertilizin by Ultraviolet Light

All irradiated and control fertilizin samples recorded in Table III were tested for univalent fertilizin by adding one drop of a test fertilizin solution (stock solution diluted to 20 per cent) to each dish after reversal had occurred in the undiluted controls. As before, the inhibition titer is that dilution of the original egg water (irradiated or control) at which agglutination was first observed upon addition of the test fertilizin. Table IV gives the data for the sample irradiated for 349 minutes. This result is similar to that obtained with the other four samples which received lower radiation doses.

The data show less inhibition (one dilution step) for the irradiated than for the control fertilizin. The difference is within the error of the method (it was found in the other four determinations as well), and may be attributed to inaccuracies in making up the dilutions, to partial inactivation of the specific combining groups of the fertilizin by the ultraviolet treatment, or more probably to incomplete reversal of the initial

TABLE IV

Ability of ultraviolet light treated S. purpuratus fertilizin to react with sperm and thus inhibit subsequent agglutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of test fertilizin to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control for sample irradiated 349 min.....	16,384	Negative	8
Egg water irradiated 349 min.....	0	Negative	4

agglutination in the control. Microscopically "complete reversal" is an ideal condition seldom attained with strong egg water. Any small unreversed clumps of sperm obscure a faint reaction by the test egg water.

Experiments on the fertilizin of the sea urchin *Lytechinus anamesis* showed that it also lost its sperm agglutinating power and became univalent when treated with ultraviolet light.

DISCUSSION

An attempt has been made to give standardized data on the X-ray inactivation of *Arbacia* fertilizin and the ultraviolet light inactivation of *S. purpuratus* fertilizin. The limitations involved in the use of "dry" sperm as a standard for sperm concentration are fully appreciated. The concentration of spermatozoa in dry sperm varies from animal to animal and with the method used in obtaining the sperm. The standard is, however, a convenient one, and has been recognized by wide application. It is accurate enough to justify its use here.

Tyler (1941) has suggested that the well known reversal of sperm agglutination in sea urchins results from a splitting of the fertilizin molecule, leaving univalent fragments attached to the sperm; and that non-agglutinating fertilizin obtained through heating or enzymatic digestion likewise involves a splitting of the molecule to form univalent

fragments. The initial rise in agglutinin titer recorded by Richards and Woodward (1915) for X-rayed fertilizin, and by Tyler (1941) for heat or enzyme treated fertilizin, are interpreted as the initial step—a splitting of the highly multivalent molecule into two or more smaller multivalent fragments—in the production of univalent fertilizin. This initial rise in agglutinin titer was not observed in the present work, although it might have been found with lower dosages.

It is shown here that fertilizin treated with X-rays or ultraviolet light loses its sperm-agglutinating power, but retains all or nearly all of its ability to combine with sperm. This indicates that a destruction of all but one sperm-combining group per molecule of fertilizin is probably not the mechanism of this inactivation. On the contrary, the data favor rather strongly Tyler's view that the original multivalent molecule is split into fragments containing but one combining group per molecular fragment.

Heat, ultraviolet light, and X-rays are denaturing agents (Clarke, 1936; Arnow, 1936), and since fertilizin is probably a protein (Tyler and Fox, 1940) it is possible that univalent fertilizin is formed as a result of incomplete denaturation of this substance. On the basis of Tyler's explanation of univalence, this would imply that in denaturation there is initially a splitting of the molecule. Such an effect has been recorded by Svedberg and Brohdt (1938) for *Helix* haemocyanin. When solutions of this material were irradiated with ultraviolet light at pH 7.4 a splitting into half and into smaller molecules occurred, followed, with prolonged irradiation, by complete coagulation. Bawden and Pirie (1937) showed that heat denaturation of the tobacco mosaic virus protein resulted in a splitting off of nucleic acid. However, a number of simpler proteins undergo little if any change in molecular weight as a result of denaturation. Sanigar, Krejci and Kraemer (1939) report no change in the absorption spectrum or sedimentation behavior of purified serum albumen after X-radiation, although treatment with ultraviolet light resulted in the appearance of substances of low molecular weight. No cleavage of the egg albumen molecule was found by Fricke (1938) through the action of X-rays, although heating caused a slight hydrolysis, the effect being greater after irradiation. Likewise, Mirsky (1938) obtained no decrease in molecular weight in the heat or acid denaturation of trypsin. Obviously, no general rule may be formulated from the work on denaturation which may be applied to the inactivation of the agglutinating power of fertilizin and the simultaneous appearance of the univalent material but Tyler's view of a fragmentation of the fertilizin molecule is supported by some of the above work and is favored here as the simplest explanation for the results obtained.

SUMMARY

1. The X-ray inactivation of *Arbacia punctulata* fertilizin reported by Richards and Woodward (1915) and by Evans, Beams and Smith (1941) is confirmed. Further data on this inactivation are presented in terms of Roentgen units, fertilizin concentration and sperm concentration.

2. It is shown that ultraviolet light is effective in the inactivation of *Strongylocentrotus purpuratus* and *Lytechinus anamesis* fertilizin.

3. *Arbacia* fertilizin which had been almost completely inactivated by X-rays, as judged by its ability to agglutinate sperm, lost none of its power to react with sperm. Thus, sperm treated with this fertilizin showed the same resistance to subsequent agglutination by untreated fertilizin as did sperm which had reversed after treatment with unirradiated (control) fertilizin.

4. *Strongylocentrotus* and *Lytechinus* fertilizins whose sperm agglutinating powers had been completely destroyed by ultraviolet light likewise retained their original sperm combining powers, as indicated by their ability to render sperm non-reactive to untreated fertilizin.

5. Tyler's (1941) view that fertilizin may be converted to a non-agglutinating, "univalent" form by splitting the fertilizin molecule into fragments, each having but one reacting group, is considered the most reasonable explanation for the results.

I am grateful to Dr. Albert Tyler for suggesting the problem, to Dr. T. C. Evans for much helpful advice, and to Dr. E. P. Little for X-raying the *Arbacia* material.

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DO SPERMATOOA PENETRATE THE MEMBRANE OF SELF-INSEMINATED EGGS OF CIONA AND STYELA?

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In 1935-36 I made many preparations (sections) of unfertilized eggs, and self- and cross-fertilized eggs of *Ciona* to find out whether spermatozoa do or do not penetrate the membrane of their own eggs. The main difficulty in reaching a decision was that the test-cells are so filled with granular materials that it is generally difficult to distinguish these, when stained, from the head of the very small spermatozoön. Nevertheless certain stains were found that could be de-stained to a point where the color of the particles in the test cells was removed leaving the stain in the heads of the sperms. Iron haematoxylin (half an hour) followed by thorough extraction gave good slides. More striking contrasts were obtained in eggs (killed in Bouin or in Carnoy solutions), sectioned, and stained in basic fuchsin (15 minutes); followed by picro-indigo carmine (six minutes), and washed in tap water.

The results showed that the spermatozoa do not as a rule pass the membrane of the eggs of the same individual. In other words, the block to self-fertilization is in the membrane.

During the winter of 1941-42 I have studied the problem again. In addition to the stains mentioned above, Ehrlich's haematoxylin (seven minutes) extracted in acid alcohol (half a minute or less), gave excellent preparations, as also did acetic-orcein (LaCour) followed in some cases by a fast green counterstain. For the study of the sections I had a better set-up than before, especially better illumination. Practically all the sections were examined in collaboration with Dr. Jack Schultz. Each case was examined by both of us. I am grateful to him for help and critical examinations.

Ciona Intestinalis

A few spermatozoa are as a rule found inside the membrane of cross-fertilized eggs, but only one or two in a section, some of them against the surface of the egg, others among the test cells. The sperm are stained

deep red after orcein or haematoxylin. It is difficult to determine whether any of the sperms are inside the test cells or merely between them; the best evidence seems in favor of the latter view. In *Ciona* the test cells form practically a continuous covering around the egg, and it might seem that the sperm are obliged to pass through them to reach the surface of the egg, but the test cells are loosely held together and it may be that sperms can pass between them. The test cells are green after the orcein and fast green, and the sperm red. They are unstained after the haematoxylin if properly extracted. The red-stained heads of the sperms, both outside the membrane and inside it, are strongly contrasted with the contents of the test cells.

The eggs that have been carefully removed from the oviduct, with no sperm added, show in sections, deeply stained particles and strands in the test cells unless the test cells are thoroughly de-stained, or are very little stained. These particles are of a different color from the sperm (orcein and fast green). The contents of the test cells are in these respects exactly like those in the self- and cross-fertilized eggs.

The selfed eggs are like the unfertilized eggs. In one or two cases a sperm has been found inside the membrane. Inasmuch as selfing may occur on a small scale such exceptional cases may be expected to occur, even in sets where no later cleavage happened; for, unless a sperm penetrated the membrane near the antipole, it might not enter the egg.

Certain precautions have to be taken with the sections of these selfed eggs. Occasionally one or more outside sperms may be dragged by the section-knife across the surface of the egg on one side. They will lie above or below the cut surface of the egg and, as a rule, there is no difficulty in detecting their origin. Slight folding of the edge of the section may give the appearance of sperm inside the egg, but such displacements are obvious.

Sections of cross-fertilized eggs show that spermatozoa may penetrate the membrane at all points of the surface, and not simply at the antipole where they also enter to fertilize the eggs. In exceptional cases it is possible that sperms may get in at other points of the surface of the egg before those that enter at or near the antipole, and if they do they may possibly enter the egg, but I have found no evidence of this. If they do, it is possible that abnormal cleavage or development would take place, but I have no grounds for such an assumption.

Styela (Cynthia) partita

At the end of the summer of 1941, five sets of eggs of *S. partita* were preserved. Records of the behavior of the remainder of the same eggs were made. The eggs were taken from the dishes in which they had

been ejaculated (along with the sperm) after 10 to 20 minutes. In some of these cases cross-fertilizations were also made to test the functioning of the sperm, and as a control of the selfed eggs. The sections were stained either in acetic orcein or in haematoxylin (Ehrlich).

1. Eggs and sperm were set free at 8 P.M. Some eggs were later preserved. None of the left-overs segmented, nor were embryos present the next morning. The stained selfed eggs showed no sperm inside the membrane, but stained sperm outside.

2. None of the selfed eggs was observed to segment, but next morning some normal tadpoles were present, presumably from eggs that cleaved late. The stained sections showed a few sperm inside the membrane both in the orcein and in the haematoxylin preparations. Some of the same eggs crossed to sperm of number (1) gave some embryos the next morning, showing that the sperm of (1) was "good."

3. None of the selfed eggs segmented. No sperms were found inside the membrane in the stained preparations with the possible exception of one sperm which was doubtfully inside.

4. Most of the selfed eggs produced abnormal embryos. Sperm were found inside the membrane in some eggs, but only a few in each case. The cross-fertilized eggs, to (3), gave 50 per cent normal tadpoles.

5. No cleavage or embryos were recorded, but the best preparations show a few sperms inside the membrane. No crosses were made.

DISCUSSION

The preceding evidence shows very clearly that in most cases no sperms are found inside the membrane of self-fertilized eggs in cases where no cleavage or embryos were observed. In the last case (5), however, a few sperm were found. Since self-fertilization does take place to a considerable extent in *Styela* it is to be expected that sperm may enter some of the eggs. Cleavage or development would be expected, in some cases at least, provided one of the sperm entered at or near the antipole. If this did not happen it is doubtful if sperm reaching the surface elsewhere would enter the egg, and it is also possible that when a sperm does enter the egg in the antipole region, the surface of the egg may then become resistant to sperm, present at another part of the egg, as is known to be the case in many other eggs.

This examination shows that after suitable staining, i.e., when the sperm are sharply marked off from the contents of the test cells, the sperm are not present or rarely present in or between the test cells after self-insemination. Whether some of the entering sperm pass through or around the test cells is unimportant for the present discus-

sion. I have observed cases where the sperms that were inside the membrane were not inside the test cells, other cases where they appeared to lie on the surface of the test cells, and a few cases where they seemed to be inside these cells. The test cells of *Styela* do not completely surround the egg, as they do in *Ciona*. These cells are often grouped in twos, threes, or more with intervening spaces. Sperm entering these spaces could pass directly to the surface of the egg. There is not the slightest evidence, even after cross-fertilization, that the test cells are filled with sperm. Without suitable staining and extraction the threads and granules might easily be misinterpreted as spermatozoa.

Styela Montereyensis

To date I have not been able to bring about spawning in this species, but abundance of eggs and sperm can be obtained from large individuals by cutting open the animal, removing the digestive tract (including the pharynx), slicing off the ovaries and their ducts, and, necessarily, the testes also and sperm ducts at the same time. A cloudy suspension is obtained, the debris is removed, the eggs concentrated by revolving the dish, the supernatant fluid poured off, or else the eggs removed to sea water. Neither cross- nor self-fertilization takes place (in January) in many cases, but at times cross- and even some self-fertilization occurs, and these are the only ones in the following account that were used to test the entrance, or failure of the sperm to enter. The eggs were killed in Bouin's solution in most cases, cut into sections, and studied under an immersion lens. Active sperm are rarely observed, whether because of the presence of mucus from the body tissues, or because the spermatozoa are sluggish in this species, or are immature. The failure to cross-fertilize seems to be due to failure of the sperm rather than of the eggs. Attempts to activate the sperm by treatment with ether, or with various salts, have failed as a rule, but in a few cases by the use of lemon juice (2.7 pH) self-fertilization took place when it did not occur in eggs fertilized in sea water or had occurred to a smaller extent. A good many preparations of the eggs of this species were examined, both in cases where no cleavage or development took place, and in cases where crossing led to a considerable amount of cleavage and sometimes 100 per cent of embryos. In the former cases no sperm were found inside the membrane. The test cells in this species, as in the other *Styela*, contain yellow-red granules and threads of material that stain, but after proper extraction these lose their color while it remains in the heads of the spermatozoa. The latter stand out conspicuously, being stained deep red (acetic orcein). The sperms here, as in the other species, are large

and can be identified by their shape as well as their color. Nevertheless, I have not found any eggs, even in those that gave a high percentage of cleavage, with sperms inside the membrane, although one sperm at least must have entered at the antipole in the eggs that cleaved. The paucity of active sperms may in this case be the explanation of failure to find any inside the membrane.

Removal of the Test Cells from Most of the Egg's Surface

In order to find out whether the presence of the test cells plays a role in inhibiting the entrance of spermatozoa into their own eggs I centrifuged (1938) the unfertilized eggs of *Ciona* for two hours at a high speed (about 3,500 r.p.m.). The test cells were driven to one pole of the egg where they form a thick mass, or a zone of clear material. The surface of the rest of the egg is brought into contact with the membrane. Such eggs were tested by selfing and crossing. It was found that they do not self but will cross. Control normal eggs were tested in each set and gave corresponding results.

The number of experiments of this kind was small and I repeated the procedure in the autumn of 1941. The first test was negative, the eggs neither selfed nor crossed. I noticed, however, that the centrifuge that I used was quite warm after two hours, and failure of the eggs to cross-fertilize was undoubtedly due to the high temperature. To avoid this I removed the machine to a cold room (4° C.), but after two hours the test cells were not moved. The low temperature had, it appears, stiffened them or produced some other effect such that they were not moved. Then a different make of centrifuge was used that did not warm up during the two hours of rotation. The results in every case were uniformly good; the selfed eggs did not segment while practically all of the crossed eggs segmented.

The results confirm the earlier ones and demonstrate that the test cells are not concerned with the block to self-fertilization. The results do not show, of course, that the block may be in the surface of the egg. Other observations or methods are necessary to demonstrate that it is the membrane around the egg and not its surface that inhibits self-fertilization. Evidence bearing on this point was obtained some years ago (1923) by cutting the membrane which sometimes allows the whole egg either to squeeze out, or to pinch off fragments of different sizes. These exposed eggs and fragments self-fertilized and segmented. It might be argued that the operation itself had affected the surface of the egg so that it lost its power to block the entrance of its own sperm, but this seems to me a rather far-fetched argument, purely theoretical, since such exposed eggs gave normal cleavage and sometimes normal embryos.

SUMMARY

A comparison of unfertilized eggs of *Ciona* with self- and cross-fertilized eggs show that spermatozoa do not, as a rule, penetrate the membrane of their own eggs. Rarely a sperm may be found inside a self-inseminated egg, which is consistent with the fact that occasionally self-fertilization occurs. Even in cross-fertilized eggs only a very few sperm are found inside the membrane of such eggs. The same statement holds for two species of *Styela* examined, except that a few more sperm are likely to be found inside the membrane of the self-inseminated eggs, which is consistent with the fact that self-fertilization is more frequent in these species than in *Ciona*.

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BEHAVIOR OF THE NURSE-CELLS OF *LITTORINA* *IRRORATA* (SAY)

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As the writer has shown (in press) the mode of development of the nurse-cells of *Littorina irrorata* is in many respects such as to indicate their homology to the atypical spermatozoa of other prosobranchs. The present paper is concerned with the behavior and history of the nurse-cells since they furnish additional support to this view.

The methods used consisted simply in the examination of temporary mounts and permanent smears of the contents of the different parts of the reproductive tract of both sexes. This material was secured with a pipette during dissection of the animals in sea water. The smears were fixed and stained by the usual methods. These were useful as a check upon the observations made on living material, for photographs and as a permanent record of the observations.

Behavior of the Nurse-cells and Spermatozoa from the Sperm Duct

The general appearance of the nurse-cells with tufts of quiescent spermatozoa attached is shown in Figure 1. Such nurse cells were found abundantly in the first (coiled) portion of the sperm duct of every male examined (July). The seminal vesicle, prostate and the distal portions of the sperm duct were entirely empty in every case. When the nurse-cells are removed from the duct and examined in sea water all of the spermatozoa are at first quiescent. Within a few minutes, however, some of them begin to show slight movements of their tails. These movements gradually increase in amplitude, and more and more of the spermatozoa begin to move in the same way until the entire group attached to a given nurse-cell is beating rhythmically. The nurse-cell is thus propelled through the water until it reaches some obstacle or until the tails of the sperm of one tuft become entangled with those of another. The latter occurs in more and more tufts the longer the cul-

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ture is maintained until finally (in about 30 minutes usually) hundreds of individual tufts have become entangled with each other. The nurse-cells themselves lie peripherally and are moved passively to and fro with the beating movements of the tufts. The three-dimensional pinwheels formed in the above manner (Fig. 4) often are seen to revolve in the liquid medium, if space permits. They may undergo progressive movement if there happen to be more sperm tufts on one side than on the other. The formation of these aggregates gives the culture a spotted appearance which is visible to the naked eye.

After some time a few of the spermatozoa of each aggregate become detached from the nurse-cells. Other spermatozoa become detached until finally all of the sperm attached to a given nurse-cell have lost contact with it. When this happens the nurse-cell set free in this way is forced peripherally by the currents set up by the moving spermatozoa. When all of the nurse-cells are free the spermatozoa still form an aggregate being completely entangled by their tails. These latter aggregates persist for some time. The heads of the spermatozoa are radially disposed around the periphery of the aggregate. After a time the spermatozoa become disentangled from the aggregate and swim freely away from it. The free spermatozoa show a marked thigmotaxis and soon become attached by their heads to any available surface but not to the free nurse-cells.

Behavior of Nurse-cells in Female

Some 200 females were dissected in order to follow the history and fate of the nurse-cells and spermatozoa. In only a small percentage of cases was a recent copulate to be found. The great majority of the females were found with empty uteri, but nearly all of them contained a large number of spermatozoa in the seminal receptacle. This organ is

PLATE I

FIG. 1. Smear from sperm duct showing nurse-cells with attached spermatozoa. Osmic vapor. $\times 579$.

FIG. 2. Smear from uterus showing nurse-cells with contained spermatozoa. A few free spermatozoa are to be seen here and there. Zenker's fluid without acetic. $\times 579$.

FIG. 3. Proximal end of spermatophore taken from uterus showing the spermatozoa agglutinated head-to-head after segregation from nurse-cells. Osmic vapor. $\times 48$.

FIG. 4. Three-dimensional pinwheel aggregate formed of many nurse-cells with attached sperm tufts. Somewhat distorted prior to fixation. Osmic vapor. $\times 48$.

FIG. 5. Distal end of same spermatophore shown in Figure 3 showing the nurse-cells segregated from spermatozoa and about to be extruded. $\times 48$.

FIG. 6. Section through female at the level of the seminal receptacle showing typical spermatozoa attached by their heads to the lining cells. $\times 5.6$.

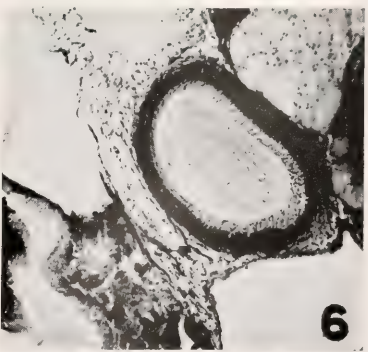
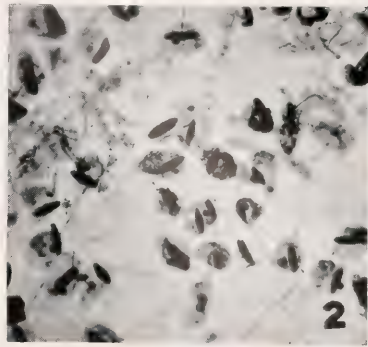


PLATE I

Figures 1 and 2 were made with a 4 mm., apochromatic objective (N.A. 0.95), Figures 3, 4 and 5 with a 16 mm., apochromatic objective (N.A. 0.30), and Figure 6 with a 32 mm., microtessar. The magnification of each figure is given in the descriptive legend.

a blindly ending sac lying posterior to the site of sperm deposition. Figure 6 shows a section through a female at the level of the seminal receptacle. The heads of the spermatozoa are attached to the epithelial cells lining the receptacle, the tails projecting into the lumen. No nurse-cells were found in the seminal receptacle. Linke (1933) found the same arrangement in the species which he studied, and he also observed sections of *L. obtusata* which included an egg as it passed the opening of the seminal receptacle into the oviduct. The egg was surrounded by spermatozoa which appeared to be streaming from the seminal receptacle, indicating that this is the site of fertilization.

In three of the females examined, the distal end of the uterus contained a cylindrical mass of semen which showed considerable resistance to breakdown when removed for study. In one case this mass contained a large number of nurse-cells with attached spermatozoa, in another case it consisted of nurse-cells and free spermatozoa, and in a third of nurse-cells only. Many of the nurse-cells in the latter copulate were swollen or otherwise distorted. In the second case cited above the nurse-cells were located at the distal end of the mass only, the spermatozoa being segregated at the opposite or proximal end, i.e., nearer the seminal receptacle (see Figs. 5 and 3, respectively).

In two of the females in which the above described sperm mass was not present the uterus contained, instead, a large number of swollen nurse-cells and a few spermatozoa. The nurse-cells were in the act of ingesting living spermatozoa, and many of them contained hundreds of sperm (Fig. 2). In many cases spermatozoa could be seen within these cells, coiling and writhing about in a large watery vacuole contained in the nurse-cell.

The spermatozoa of *L. irrorata* show a pronounced head-to-head agglutination reaction when not attached to nurse-cells. This is true of segregated sperm taken from the seminal receptacle as well as of those taken from the proximal end of a recent copulate. This agglutination can be seen in Figure 3 which represents the proximal end of such a copulate.

DISCUSSION

The most remarkable aspect of the history of the nurse-cells lies in their behavior when withdrawn from the sperm duct. The formation of the three-dimensional pinwheels is similar in many respects to the formation of clumps of apyrene and eupyrene spermatozoa described by the writer (Woodard, 1940) for *Goniobasis*: 1. Both are brought about by the sticking together or entanglement of spermatozoa by their tails. 2. Both result in the formation of temporary aggregates. 3. In both

cases the atypical components play out their role and from this time on show no further ordered behavior towards the spermatozoa. 4. In *Goniobasis* a tail-to-tail aggregation of the spermatozoa is a condition intermediate to their final disposition (in the seminal receptacle). This is presumably also the case in *L. irrorata*. 5. In both cases these aggregates eventually disperse, and the free spermatozoa show a tendency to wander away from the site of aggregation, and a marked thigmotaxis. 6. In both cases the spermatozoa eventually become sharply segregated from the atypical structures and reach a seminal receptacle where they are stored. 7. In both cases the atypical structures undergo breakdown and extrusion after their functional phase has passed. 8. Finally, in both cases the atypical structures act as an aid in the aggregation. In the case of *Goniobasis* as I have shown experimentally, the atypical spermatozoa are necessary. In the case of *L. irrorata*, it may be emphasized, both nurse-cells and spermatozoa may be present separately, but when not attached the spermatozoa aggregate head-to-head (agglutinate) rather than by their tails. This would seem to indicate that an atypical structure is necessary for tail-to-tail aggregation.

The observations of Tyler (1940) may be of great significance in this connection. He has shown that in *Megathura crenulata* two types of sperm aggregation occur: one is a head-to-head aggregation which he regards as primarily a dilution effect; the other is a tail-to-tail agglutination which is effected by a substance present in the jelly layer of the egg. The existence of such a specific tail-to-tail agglutinin suggests that, in the case of both the apyrene spermatozoa of *Goniobasis* and the nurse-cells of *Littorina*, we may be dealing with structures which can in some way produce the same effect upon the spermatozoa as the substance from the jelly layer of the egg in Tyler's experiments. It may thus be possible to arrive at a biochemical proof of the essentially egg-like character of the atypical spermatozoa of prosobranchs where the morphological proof remains more or less indecisive.

SUMMARY

The nurse-cells of *L. irrorata* with tufts of sperm attached are moulded in the male into rudimentary spermatophores and are then passed to the female. In the uterus the sperm become detached from the nurse-cells and migrate to the seminal receptacle where they are stored. The nurse-cells then ingest any remaining spermatozoa, break them down, and are themselves eventually extruded. Nurse-cells with tufts of sperm in the sperm duct show an aggregation reaction which leads to the formation of three-dimensional pinwheels. The tufts of

sperm are attached by their tails to each other, and the nurse-cells lie radially disposed. These aggregates disperse first by detachment of the nurse-cells, leaving the sperm attached by their tails, then by disentanglement of each of the sperm. The free sperm then show a thigmotaxis. Sperm not attached to nurse-cells (from seminal receptacle or from a copulate in which segregation has already occurred) show the usual head-to-head agglutination reaction. The behavior of the nurse-cells and spermatozoa is viewed as a mechanism whereby the latter are segregated from the former. This behavior is compared with the clumping of the apyrene and eupyrene spermatozoa of *Goniobasis*.

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